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Ultra-Sensitive Detection of Prion Protein in Blood Using Isothermal Amplification Technology

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The detection of pathologic prion protein that is implicated in transmissible spongiform encephalopathies is necessary to diagnose disease. Presently, the Western blot or ELISA are used to test the brain stem in cattle for the presence of PrPsc after proteinase K digestion of normal, cellular prion (PrPc) before admission of these animals into the food chain. However, infected animals and humans cannot be diagnosed in the pre-clinical stage of infection. Because routinely used prion detection methods can identify PrPsc in brain where quantities are high but cannot detect PrPsc in blood where levels are low, a lack of sensitivity of current assay methods is likely the explanation. To address this, we have exploited a real-time immuno-polymerase chain reaction method (IPCR) that couples serologic detection of protein with the amplification ability of PCR and applied it to the detection of prion protein. The method has been optimized for maximum sensitivity through a number of procedural modification and background reduction strategies. Efforts were extended to develop a new method called RNA polymerase immunodetection (RAPID) that is similar but uses an RNA polymerase to produce RNA transcripts at isothermal temperatures and a magnetic bead solid support. Results have shown the sensitivity of IPCR to be 100 attograms/mL of recombinant prion protein, and detection levels using scrapie infected hamster brain homogenates down to 10-100 infectious units. This sensitivity is unmatched by other assays and approaches the sensitivity believed to be required for pre-symptomatic detection of prion protein in blood. The RAPID method has been developed using a number of the parameters that were optimized in the IPCR method; however, its sensitivity was shown to be only 150 pg/mL.

14. SUBJECT TERMS
Detection of prion in blood, PrPsc, PrPc, isothermal, amplification, PCR, IPCR, RAPID

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INTRODUCTION:

The identification of prion diseases prior to clinical symptoms or death would address several critical issues. First, a screening method to detect infection would assure the safety of blood targeted for transfusion. Scientific reports indicate that prion diseases can be transfusion-transmitted via blood from infected humans and animals many months prior to the appearance of symptoms. Second, there is growing public concern about the safety of the food supply, knowing that infected beef has been implicated as the cause of variant Creutzfeldt-Jakob Disease (vCJD) in Europe. An effective screening test for cattle (and deer/elk) would ease concern for the general public. Third, there has been an enormous, negative economic impact on the food industry in Europe due to the slaughter of thousands of cattle because of concern of infection, and additional concerns in the United States because of beef importation and exportation restrictions. The first case of mad cow disease (BSE) was identified in the US (Llewelyn et al., 2004) in 2004, and a second case in the summer of 2005. It is certain that a substantial economic impact may be realized if more cases of mad cow disease in the United States should occur. Although the USDA is testing about 300,000 cows per year, there are concerns about the adequacy of the tests used (samples from the last cow were sent to Europe for testing). It is envisioned that a suitable blood or urine screening test to detect prion infection in cattle, deer, elk, and other species, would eliminate the need to slaughter uninfected animals. Fourth, the US military and other US organizations have concern about possible undetected prion infection in US personnel and their families who were stationed in Europe during the prion epidemic that occurred in the 1980s.

Our rationale for suggesting that amplification techniques be applied to the early detection of prion diseases is comparable to past experience with HIV diagnostics. Prior to the development of nucleic acid tests, HIV infection could not be detected by antibody tests at less than 3 weeks after infection. However, the use of nucleic acid amplification techniques proved that low levels of an HIV diagnostic marker could indeed be detected, and the use of these tests has revolutionized diagnostics for early detection of a number of infectious agents. Consequently, the nucleic acid tests for HIV (and HCV) are recognized by the US FDA and are now used to test all blood units targeted for transfusion. The situation for prion may not be dissimilar; i.e., markers are present but cannot be identified due to the lack of tests with exquisite analytical sensitivity. This is supported by the facts that the infectious prion protein can be detected serologically in tissues (brain) that have high levels of prion, and that blood has been shown to contain the infectious unit. However, prions cannot be detected in blood using the same tests. Accordingly, this can be explained by the lack of sensitivity of currently used tests. However, amplification methods are applied to the detection of nucleic acids, and infectious prions are devoid of nucleic acid. Therefore, in order to apply these techniques, nucleic acid amplification techniques must be coupled to the serologic detection of proteins (antigens) such that are used for antigen assays.

Detection of the pathologic prion protein that is implicated in transmissible spongiform encephalopathies (TSEs) is necessary to diagnose the disease. Presently, before cattle products are admitted into the food chain, the Western Blot or ELISA in an antigen detection format are used to test the bovine brain stems for the presence of pathologic prion (PrPSc) after Proteinase K (PK) digestion of normal, cellular prion (PrPC). An animal in the end stages of disease (40 - 72 weeks after infection) will be detected by these methods; however, infection will not be detected by these methods during preclinical stages of prion infection (1-20 weeks). The immuno-polymerase chain reaction (IPCR) and a related method – the RNA-polymerase immunodetection method (RAPID), are techniques whereby the exponential amplification ability of PCR is coupled to antibody-facilitated protein detection in an enzyme linked immunosorbent assay (ELISA) format; these can be configured in tubes, microtiter plate wells, or using magnetic beads as the solid support. The RAPID method is similar to the immuno-PCR method except that the final step of nucleic acid amplification is by an RNA polymerase that is conducted at isothermal incubation temperatures.

BODY:

Our objective was to develop an innovative amplification technique and show its application for detecting ultra-low levels of prion protein in blood and urine. The specific aims were to: (1) Develop a prototype, ultra-sensitive RAPID method for prion protein detection and define its increased sensitivity over currently used prion assays; (2) challenge the method for its femtogram/ml or below sensitivity in prion-spiked blood and urine, and in blood from infected animals, and (3) standardize the method for reproducibility and readiness for transfer to a commercial entity.

We have exploited the immuno-PCR method (IPCR) of Sano et al., 1992, with substantial modifications, for the detection of ultra-low levels of prion protein (and HIV-1 p24 Ag) and have published our findings (Barletta et al., 2004; Barletta et al., 2005). The modifications which were applied to the IPCR method resulted in attainment of a sensitivity unmatched by other methods for prion protein, and constitutes a significant accomplishment. Select data are presented below. For the RAPID method, which is similar to the IPCR method in many of its parameters but uses a different amplification strategy and magnetic beads as the solid support, we have been successful in showing proof of principle and
have obtained sensitivity down to 150 pg/mL. It is certain that this sensitivity can be improved, but as of present, experiments to optimize the method did not result in improvement. Select data on the RAPID method development are presented below. Thus, we have accomplished our specific aim #1 by developing an amplification method having sensitivity up to a million fold greater than other prion methods using I-PCR, and have developed the prototype RAPID method with a good sensitivity.

We partially accomplished specific aim #2 by obtaining data showing the detection of PrPSc in PK-digested scrapie infected hamster brain homogenates at 10 - 100 infectious units (down to 10,000 PrPSc molecules). Because capture antibodies that we were using became unavailable during the last 6 months of the grant, the method could not be used to spike prion into human blood and urine or for testing blood from infected hamsters. All of these media have been obtained and if suitable antibodies become available, we are planning to complete this aim.

We partially accomplished specific aim #3 which included the standardization of the method for reproducibility and its readiness for transfer to a commercial entity. The IPCR method has been standardized to the point where 75% of runs are acceptable using multiple controls (Section 4.4 of the Discussion in Barletta et al., 2005); we believe this is an acceptable level for transfer to a company who specializes in manufacturing diagnostic assays. Lessons learned in accomplishing this for IPCR were not applied for the RAPID method because its sensitivity was sub-optimal.

As related to the specific tasks proposed, we have partially met the requirements for Task #1 by developing the RAPID method and defining its sensitivity. However, efforts are needed to improve sensitivity so that it reaches levels obtained by IPCR. The antigen capture ELISA using magnetic beads has been developed, all reagents for the RAPID method have been applied, the amplification reagents have been titrated, and real-time measurements have been successful. For Task #2, efforts to address prion extraction methods are progressing by assessing specific antibodies that can differentiate PrPc from PrPsc (data presented below, see Figure 5), rather than using PK treatment; user-friendliness and comparison to ELISA has revealed that the IPCR and RAPID methods are more demanding than ELISA. Several ideas to increase the user-friendliness of these amplification strategies have been devised (confidential information). Task #3 was not attempted because of the unavailability of suitable antibodies to continue with the IPCR and RAPID methods; however, all sample materials, including blood from infected animals, were obtained. Task #4 (standardization for transfer to a commercial source) has been completed.

Figure 1 shows the IPCR standard curve using recombinant PrP; as shown, a dose response was observed from 1ug to 1 fg/ml. Although 100 attograms/ml was detected, this was not shown to be reproducible to the degree we required. Nevertheless, it shows the potential of IPCR to detect in the sub-femtogram/mL level. Figure 2 depicts the detection of PK-digested brain homogenates down to 70 fg/mL (10-100 infectious units).
Figure 1. Standard curve for recombinant PrP<sub>C</sub> using IPCR.
For the RAPID method, several of our modifications which we had shown to be significant improvements in the IPCR method were implemented in the RAPID protocol.

These modifications include:

1. Use of the same animal species capture and secondary biotinylated antibody (to decrease inter-species non-specific cross reactivity),
2. Use of a DNA:Stabilcoat blocking reagent composed of a “DNA Blocking Reagent” (Roche Diagnostics; Indianapolis, IN) combined 1:1 with “Stabilcoat” (Surmodics, Eden Prairie, MN) designed to minimize non-specific protein and DNA interactions in nucleic acid hybridizations (Roche Diagnostics; Indianapolis, IN). This blocking buffer was tested in the magnetic bead assay and found to be superior to Seablock, Superblock (Pierce Co., Rockford, IL), and 5% BSA for the reduction of non-specific background.
3. Addition of “FcR Blocking Reagent” (Miltenyi Biotec; Auburn, CA) to the secondary antibody dilution to block antibody Fc receptor non-specific interactions,
4. Addition of 10 mM EDTA and 5 Units/mL sodium heparin to all buffers and wash reagents to minimize non-specific binding caused by molecular ionic interactions,
5. Addition of 2 extra blocking steps (one after SA-HRP addition) with DNA:Stabilcoat blocking reagent and pre-PCR (described by Nunc-Nalge Corp. in a TechNote described at (http://www.nunc.nalgenuncc.com/resource/technical/nag/DP0031.htm).
6. Multiple biotinylation of the DNA template to increase efficiency of binding to the linker streptavidin molecule.
Research accomplishments associated specifically with the RAPID protocol include:

1. Determination of the most sensitive fluorescent dye (SYBR Green I, SYBR Green II, or SYBR Gold) detection of transcription products using real-time technology.

In these experiments, 3 different fluorescent dyes: SYBR Green I, SYBR Green II, and SYBR Gold (Molecular Probes; Eugene, OR) were tested at different dilutions using a pGEM control vector plasmid (Promega; Madison, WI) as a DNA template (2743 bp) containing the T7 promoter. Reagents from the T7 Megascript Kit (Ambion Inc.; Austin, TX) were used in these studies. Ambion, Inc. claims that the reagents in this kit will produce mg amounts of RNA products from ug quantities of DNA template. Figure 3 shows that SYBR Green II exhibited higher relative fluorescence units (RFU) than SYBR Gold (i.e., 500-6,000 vs 200-1,700 RFU, respectively) of transcript products detected in real-time by the iCycler system (Bio-Rad Laboratories; Hercules, CA). SYBR Green I exhibited extremely low fluorescence in comparison to both SYBR Gold and SYBR Green II and was not considered for further studies (data not shown). In later experiments SYBR Green II exhibited even higher RFU than SYBR Gold ranging from 1,000-10,000 when used at lower dilutions (1:5,000) for detection of transcript products (data not shown). SYBR Green II was therefore selected as the optimal fluor for further experimentation using the RAPID method.
Figure 3: Real-time amplification plots of fluorescent transcripts generated by T7 RNA polymerase using various amounts of pGEM template (1-1000 ng) for T7 transcription with either SYBR Gold (a) or SYBR Green II (b) at a 1:20,000 dilution.
Fluorescent transcripts were verified by gel electrophoresis and the results were identical to real-time data with detection of transcripts down to 1 ng using SYBR Green II at 1:20,000 and 1:5,000 dilutions (Figure 4 a and b, respectively). Additionally, fluorescence was visually detectable by UV illumination from transcript products in the reaction tube (Figure 4: c and d, respectively). The ability to detect fluorescence directly in the reaction tube may allow the test to be performed without the need for sophisticated detection equipment.

2. Development of the magnetic bead indirect ELISA protocol using HIV-1 anti-p24 antibodies and HIV-1 p24 Ag as the target analyte.

The magnetic bead indirect ELISA protocol was developed using HIV-1 p24 Ag as the analyte because the reagents used in the method are readily available in our laboratory and are much less expensive to use during method development. The optimized protocol for the magnetic bead ELISA assay is described below:

a. Incubate equal amounts of biotinylated (rabbit) secondary anti-p24 Ab (Perkin Elmer Life Sciences, Inc., Boston, MA) with various dilutions of quantified HIV-1 p24 Ag frp, infected culture supernatants (diluted in lysis buffer: 0.5% Triton-X, PBS) for 1 hr, RT;

b. Add 50 uL of the pre-incubated (Ab-Ag from a) to 50 uL of 10^7 Dynal paramagnetic beads (Dynal Biotech, Oslo, Norway) coated with 50 ug each of 13B6 and 13G4 mouse monoclonal anti-p24 antibodies (Institute of Human Virology, Baltimore, MD) for 1 hr, RT. Wash magnetic beads 3X.

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**Figure 4 a & b.** UV illumination of RNA transcripts.  
**Electrophoresis of RNA Transcripts.** Starting pGEM template amounts were 100, 10, 1, 0.1, and 0 ng in Lanes 1, 2, 3, 4, and 5, respectively. SYBR Green II was used at 1:20,000 (a), and 1:5,000 (b) dilutions, respectively. (m= molecular weight marker).  
**UV illumination of RNA Transcripts.** Starting pGEM template amounts were 100, 10, 1, 0.1, and 0 ng in Lanes 1, 2, 3, 4, and 5, respectively. SYBR Green II was used at 1:20,000 (c), and 1:5,000 (d) dilutions, respectively. (m= molecular weight marker)
c. Add 50 μL streptavidin-HRP (KPL: Gaithersburg, MD) diluted to 0.2 ug/mL in 1:10 IPCR block buffer (Barletta et al., 2004) for 1 hr, RT. Wash magnetic beads 3X. Wash 3X with PBS, 0.5% Tween-20 (WB).

d. Add 50 μL TMB (Perkin Elmer Life Sciences, Inc., Boston, MA) for 30 min, RT. Remove solution from magnetic beads and read optical density.

Sensitivity of detection was down to 150 pg/mL with a threshold signal to noise (S/N) ratio of >2.0. The average S/N ratio from replicate experiments for 15 ng/mL, 1.5 ng/mL, 150 pg/mL, and 15 pg/mL was 14.9, 6.7, 2.2, 1.2 respectively (data not shown). This indicates a maximum sensitivity of 15 pg/mL, although reproducibility is less than optimal because of the low signal to noise (1.2).

To address concerns about the use of PK treatment on the front-end of these analytical methods for prion detection, as proposed in Task #2 as prion extraction methods, we have assessed the use of combinations of antibodies to differentiate PrPc from PrPsc. The rationale is based on the fact that PrPsc is present in aggregate form, while PrPc is present in monomeric form. Therefore, PrPsc aggregates contain multiple binding sites for the same epitope, while PrPc contains only one. If capture and detection antibodies target the same or similar epitopes of the prion molecule they will react with PrPsc aggregates but not PrPc monomers, since the capture antibody has already bound the single PrPc epitope leaving it unavailable for the detection antibody. An prototype assay (MDS) was developed, in conjunction with PeopleBio company in Korea, that showed encouraging results. A number of assay parameters were investigated to optimize the differentiation, including the concentration and variety of antibodies, avidin, biotin, and blocking buffers. Select data are presented below:

Figure 5: Optimization of secondary antibody concentration for competitive binding principle

* Objectives: Test whether the optimized condition would work with normal human plasma (only monomeric PrPc). The recombinant human PrP 23-231 (full length, also contains aggregate forms of PrP) was used as control. Epitopes for 308 and 3F4 are very close spatially, therefore capture and detection antibodies compete for binding monomers, whereas aggregate forms possess multiple antibody binding sites.

* Results:
The Non-Specific Background (NSB) signals from both Control and the MDS ELISA were below OD 0.1 (Fig. 5)
Only recombinant human PrP showed a positive signal in the MDS ELISA. Signal from plasma showed similar OD as did NSB, confirming that PrPc in normal human plasma exists as a monomer, and recombinant PrP is present in aggregate form.

KEY RESEARCH ACCOMPLISHMENTS:

Progress during the 2nd year has been less than expected because of several factors: First, Dr. Barletta, the main investigator for this effort, accepted a new position and left in November 2004, ending her efforts on this project. Her replacement, Ms. Julie Giles, began in January 2005 and required 4 months to learn the techniques. Second, the prion capture antibodies that proved to be excellent for attaining the high degree of sensitivity in the IPCR and RAPID methods have been depleted and are not replaceable. Hence, a number of months were required to procure and assess several other antibodies that could meet the requirements. Several commercially-available antibodies were good candidates, but even the best did not produce the sensitivity obtained with the original ones.

Key research accomplishments during the past 2 years are listed below:

- Successful implementation of an amplification protocol (IPCR) for the detection of prion protein (Barletta et al., 2005, attached) using microwell plates as a solid format. We have shown that this novel method can attain sensitivities for recombinant PrPsc in the attogram/mL range and in diluted scrapie infected hamster brain homogenates to 10-100 infectious units; this level of sensitivity is unmatched by all other reported methods for prion detection. We have shown that this level of sensitivity is 1 million times more sensitive than our own ELISA for prion detection, and 1,000 to 10,000 more sensitive than other methods used for prion detection (Barletta et al., 2005, Gofflot et al., 2004, Biffiger et al., 2002).
- Using the IPCR method, which is similar to the RAPID test, we have gathered important information to accelerate the work with development of the RAPID protocol using magnetic beads as a solid support. The use of the same blocking buffers and wash reagents, as well as a number of experimental parameters, have been applied to the RAPID protocol. At present, the sensitivity of detection using the RAPID method is approximately 150 picogram/mL. This sensitivity of detection is comparable or higher than that described in most protocols in the literature (Kiselev et al., 1999; Kourilov et al., 2002) which typically attain nanogram/mL levels of analyte detection; however, it is not great enough to offer the necessary levels for pre-symptomatic detection (~1fg/mL).
- The fluorescent dye (SYBR Green II) has been successfully applied in the RAPID method and we have shown an increase in the detection of amplified transcripts 3.5 times (6,000 RFU/1,700 RFU for 100 ng pGEM template) over SYBR Gold and SYBR Green I fluorescent dyes.
- We have obtained a number of biological media (e.g., urine, and blood) from prion infected hamsters to be used for spiking experiments and for challenges of our methods for detection of prion in future studies.

REPORTABLE OUTCOMES:

Publications:
CONCLUSIONS:

From this award, we have developed a novel amplification method (IPCR) that has shown unparalleled sensitivity for detecting prion protein in brains from infected hamsters. Using a microwell as the solid support, we have been able to show that real-time IPCR detects recombinant hamster PrPSc down to 100 attograms/mL concentrations, and when using PK-digested scrapie infected hamster brain homogenates (10%) diluted from 10⁻¹ to 10⁻⁹, a quantitative dose response was observed that detected between 10-100 infectious units of prion protein; this has recently been published (Barletta et al., 2005). This publication, along with an earlier publication using real-time IPCR for the ultra-low detection of HIV p24 antigen, attest to the development of an ultra-sensitive analytical method that can attain the levels necessary for the pre-clinical identification of prion infection; i.e., 10 infectious units per mL (Brown et al., 2000; MacGregor, 2001). It is clear that this method outdistances all other reported methods.

The ultra-low level of sensitivity obtained by IPCR for prion detection has not yet been attained for the RAPID method. However, much of the background work involving optimized reagents for the RAPID method has been determined by our experimental work with the IPCR method. Many of the procedural steps (use of the same antibodies, blocking reagents, and wash buffers) were translated to the ELISA portion of the RAPID method using a magnetic bead solid support format. In addition, we are addressing the replacement of PK treatment by assessing combinations of antibodies in order to differentiate PrPSc from PrPSc. Results have been encouraging, but more work to increase sensitivity and user friendliness is needed.

Regardless of which method attains the highest degree of sensitivity, the next steps are to (1) challenge the method for detection of prion in spiked body fluids, and (2) assess the ability of the IPCR method to detect prion in blood from infected animals.

REFERENCES:


APPENDICES:

Detection of ultra-low levels of pathologic prion protein in scrapie infected hamster brain homogenates using real-time immuno-PCR

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Abstract
Pathologic prion protein (PrPsc), implicated in transmissible spongiform encephalopathies, is detected by antibody-based tests or bioassays to confirm the diagnosis of prion diseases. Presently, the Western blot or an ELISA is officially used to screen the brain stem in cattle for the presence of PrPsc. The immuno-polymerase chain reaction (IPCR), a technique whereby the exponential amplification ability of PCR is coupled to the detection of proteins by antibodies in an ELISA format, was applied in a modified real-time IPCR method to detect ultra-low levels of prion protein. Using IPCR, recombinant hamster PrPc was consistently detected at 1 fg/mL and proteinase K (PK)-digested scrapie infected hamster brain homogenates diluted to 10−8 (approximately 10–100 infectious units) was detected with a semi-quantitative dose response. This level of detection is 1 million-fold more sensitive than the levels detected by Western blot or ELISA and poises IPCR as a method capable of detecting PrPsc in the pre-clinical phase of infection. Further, the data indicate that unless complete PK digestion of PrPc in biological materials is verified, ultrasensitive assays such as IPCR may inaccurately classify a sample as positive.

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Constantine, N; Edelman, D; Barletta, J; Highsmith, E. “Approaching the detection of one infectious unit of prion protein using IPCR.”

Approaching the Detection of One Infectious Unit of Prion Protein using Immuno-PCR
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Background: The detection of the pathologic prion protein (PrPsc), the causative agent of transmissible spongiform encephalopathies, is required for the diagnosis of prion diseases.
Presently, the Western Blot (WB), enzyme-linked immunosorbent assay (ELISA), and rapid strip tests are used to test nervous tissue in humans and cattle for the presence of the pathologic prion following death. The development of a sensitive assay that can detect femtogram/mL levels of the abnormal prion protein may be required to detect prion in blood before death, and would be invaluable for protecting the blood supply and the food chain.

Methods: The immuno-polymerase chain reaction (IPCR), a technique that couples the serologic detection of proteins in an ELISA format to PCR that offers exponential amplification was developed. We incorporated a modified real-time IPCR method to detect hamster recombinant PrP\textsuperscript{C}, and proteinase K (PK) -digested scrapie infected hamster brain homogenates that were diluted down to 1 infectious unit (IU). The antibodies for detection included one of two monoclonal capture antibodies (against the N-terminus or the C-terminus), and a biotinylated 3F4 as the detector antibody. The amplifiable target was a biotinylated DNA molecule, and amplicons were detected in real-time using an i-Cycler (Biorad).

Results: The IPCR method detected recombinant PrP down to 100 attogram/mL. When used to test PK-digested scrapie infected hamster brain homogenates, diluted from $10^{-2}$ to $10^{-8}$ (down to 1-10 infectious units), it exhibited a semi-quantitative dose response. The level of detection was 1 million-fold more sensitive than levels attained by ELISA. By using the two different capture antibodies, it was confirmed that the ultrasensitive IPCR method will require that normal PrP\textsuperscript{C} be removed before assessing the presence of PrP\textsuperscript{sc}.

Conclusions: IPCR has exhibited an unparalleled sensitivity, detecting levels of PrP\textsuperscript{sc} in the range required to detect infection in blood and in the pre-clinical phase of infection. Further, our study indicates that unless PK digestion of the brain homogenate is verified to remove all PrP\textsuperscript{C}, highly sensitive assays such as IPCR may inaccurately classify a sample as positive.