

THESIS

DETECTING THE TEMPORAL STATUS OF BLOOD-BORNE PRIONS IN
TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHY-INFECTED HOSTS

Submitted by

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ABSTRACT

DETECTING THE TEMPORAL STATUS OF BLOOD-BORNE PRIONS IN TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHY-INFECTED HOSTS

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are infectious, fatal neurodegenerative diseases with a protracted subclinical disease state spanning months to years. Prion diseases develop when the normal cellular prion protein (PrP^{C}) undergoes a conformational change into an aberrant, disease-causing, isoform ($\text{PrP}^{\text{Sc}}/\text{PrP}^{\text{res}}/\text{PrP}^{\text{D}}$), which aggregates into amyloid fibrils. Prions are unique from all other infectious diseases in that they lack nucleic acid. Prion diseases are known to naturally occur in cattle, sheep, mink, cervids, and humans; however, the exact mechanisms of transmission are unknown. Sufficient infectious prions to transmit and cause disease are known to be present in tissues and bodily fluids of all TSE-infected mammals during clinical and subclinical stages of disease. Extensive extraneural PrP^{Sc} -deposition has been observed in chronic wasting disease (CWD)-infected cervids and transmissible mink encephalopathy (TME)-infected hamsters and is very similar to what has been described for variant Creutzfeldt-Jacob disease (vCJD)-infected humans. Importantly, blood taken from humans and animals lacking overt clinical symptoms is capable of transmitting disease through transfusion.

In this thesis we set out to answer questions regarding hematogenous prions: 1) How long does it take for prions to enter the blood after initial TSE exposure? 2) Are hematogenous prions present in all animals infected with CWD and TME? and 3) Does the route of prion entry affect the temporal status of hematogenous prions.

To answer these questions, we analyzed longitudinally-collected whole blood samples from TSE-exposed animals by a modified version of the highly sensitive *in vitro* amyloid-amplification assay “real-time quaking-induced conversion” (RT-QuIC) we termed whole blood (wb) RT-QuIC. Longitudinal whole blood samples (15 minutes post exposure-terminal disease) were collected from experimental CWD-exposed (oral, aerosol, and intravenous inoculation) white-tailed and Reeves’ muntjac deer and TME-exposed (extranasal inoculation) Syrian hamsters.

We detected PrP conversion-competent amyloid in the blood of 100% of infected animals as early as 15 minutes post inoculation throughout terminal clinical TSE disease. These results were observed for all inoculation routes. Furthermore, we observed the presence of prions in the blood in two phases—a primary and secondary prionemia.

The results of this work suggest that: 1) inoculated prions traverse mucosal barriers and enter the blood within 15 minutes of exposure; 2) the route of inoculation has little effect on the temporal status of prions in the blood; 3) there are two distinct phases of prionemia representing the initial inoculum (primary prionemia) and *de novo* host-generated prions (secondary prionemia); and 4) the observed characteristics of prionemia can be recapitulated in various TSE-host combinations and may recapitulated the extraneural pathogenesis of human TSEs.

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Rudyard Kipling once wrote:

[...] If you can dream - but not make dreams your master,
If you can think - but not make thoughts your aim;
If you can meet with Triumph and Disaster
And treat those two imposters just the same [...]. (9-12)

Those words from the poem “If” by Rudyard Kipling have stood out to me since I first heard it at my high school graduation. Those four lines are, in my opinion, the essence of what it means to be a scientist: finding a balance between dreams and reality, between failure and success. These lessons have been also been taught to me through the many friends, colleagues, and mentors over the years that have helped get me where I am today.

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DEDICATION

I would like to dedicate this work to:

My wife, Olivia Elder. Thank you so much for everything. Thank you for your support during stressful times. Thank you for your patience with all of my late night studying. Thank you for celebrating with me when I passed an exam or finished a paper. But most of all, thank you for your encouragement during this endeavor.

My family—Tom, Denise, Mary, and Jack—and in-laws—Rebecca, Jeff, Haley, and Madison. Thank you for encouraging me in my pursuits and to not to give up when the going got tough. I would especially like to thank my mom and dad for teaching me a strong work ethic and, more importantly, how to be a good person with good values. Thanks for everything.

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INTRODUCTION

Protein misfolding and amyloid formation:

The proper folding of proteins is essential in all life; so much so that there are conserved mechanisms spanning from the simplest bacteria to complex mammals to ensure proper protein folding. While these mechanisms function accurately the majority of the time, protein misfolding does occur. Intricate cellular responses are usually sufficient in correcting the misfolding, or targeting the protein for degradation [1]. However, aggregation of misfolded proteins into well-ordered, insoluble, fibrils—known as amyloids—is also possible and can have beneficial effects or, more commonly, be deleterious to the host. An example of beneficial amyloids are the prion-like amyloids formed in yeast, such as URE3 and PSI, which aid fungal growth in nutrient poor environments and help regulate the translation of specific genes, respectively [2; 3]. The generation of beneficial aggregates is not limited to simple eukaryotes though; an example in more complex eukaryotic cells is the aggregation of the mitochondrial protein MAVS, which function to activate the innate immune response to viral infection [4]. Despite the existence of beneficial amyloids, many amyloids have been implicated in a large number of disorders, including many neurodegenerative diseases (Table I.1).

When certain proteins misfold, their tertiary structures adopt uncharacteristic conformations that are abundant in beta-sheets (Fig. I.1), which results in self-aggregation and amyloid formation. Amyloid polymerization occurs as the beta-strands of each misfolded protein monomer align to create the cross-beta sheet quaternary structure of the amyloid fibril. It is this cross-beta sheet structure that allows the detection of many amyloids through the use of dyes (e.g. Thioflavin T and Congo Red), Fourier transform infrared spectroscopy (FTIR), and

transmission electron microscopy (TEM) [5; 6]. A common hallmark of amyloids, despite the different originating proteins, is the resistance to protease digestion resulting from the dense packing of the cross-beta sheet structure [7-9]. The inability to degrade insoluble amyloids results in their continued accumulation, eventually leading to the development of disease; however, the exact mechanisms of amyloid-induced pathology are unknown.

Table I.1. Diseases involving amyloids

Disease	Normally folded protein	Aberrant folded protein	Tissue(s) of aggregation	Protein aggregates detected	Cause
Prion diseases	PrP ^C	PrP ^{Sc}	Systemic, but primarily central nervous tissue	PrP ^{Sc} deposits	Sporadic Genetic Acquired
Huntington's Disease (HD)	Huntingtin protein (Htt)	Mutant Htt (mHtt) aggregates	Central nervous tissue	mHtt inclusion bodies	Genetic
Alzheimer's Disease (AD)	Amyloid precursor protein (APP)	Amyloid beta peptides (A β)	Central nervous tissue	A β plaques	Sporadic Genetic
Parkinson's Disease (PD)	α -Synuclein	α -Synuclein aggregates	Central nervous tissue	Lewy bodies	Sporadic Genetic
Amyotrophic lateral sclerosis (ALS)	Superoxide dismutase (SOD1)	Mutant SOD1	Central nervous tissue	Mutant SOD1 aggregates	Sporadic Genetic
Tauopathies	Tau	Tau aggregates	Central nervous tissue	Neurofibrillary tangles (NFTs)	Sporadic Genetic
Amyloid A (AA) amyloidosis	Serum amyloid A (SAA)	N-terminal fragment of SAA (AA)	Systemic due to chronic inflammation such as rheumatoid arthritis	AA deposits	Sporadic
Type II diabetes mellitus	Islet amyloid polypeptide (IAPP)	proIAPP and IAPP	Pancreas	IAPP amyloids	Sporadic

There are many hypotheses to explain the development of amyloidogenic diseases. Some amyloidoses, such as amyloid-associated kidney disease and type II diabetes mellitus (DM), have been observed to cause disease through the disruption of tissue architecture in various organs [10; 11]; however, it is suspected that many amyloidogenic disorders (e.g. Alzheimer's disease (AD), Parkinson's disease (PD), rheumatoid arthritis, and prion diseases) cause disease through calcium dysregulation and mitochondrial dysfunction leading to the release of reactive oxygen species [12; 13]. For amyloidoses involved in neurodegenerative diseases, protein aggregates have been observed as both intra- and extracellular fibrillar aggregates indicating a possible toxic extracellular effect. Additionally, a new hypothesis has emerged implicating prefibrillar intermediates as the culpable agents of neurotoxicity in various neurodegenerative amyloidoses [14-16].

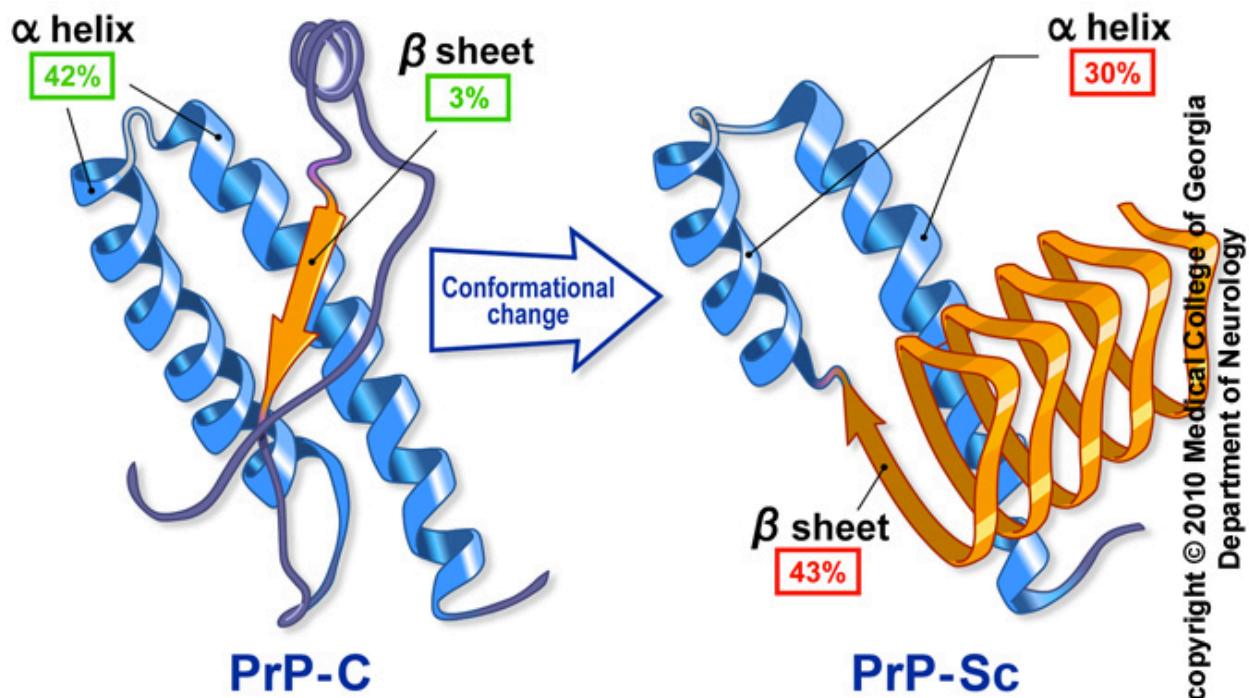


Figure I.1. Conformational change from PrP^C to PrP^{Sc}
<http://curtis.wawiki.wikispaces.net/Bradley+Waddell>

In addition to the various ways amyloidoses cause disease, the origin of amyloid formation can differ between diseases. Amyloid generation can be initiated through genetic inheritance (PD, AD, Huntington's disease (HD), Amyotrophic lateral sclerosis (ALS), Fatal Familial Insomnia (FFI)), spontaneous means (sporadic Creutzfeldt-Jacob disease (sCJD)), iatrogenic measures (CJD), and infection (vCJD, scrapie, bovine spongiform encephalopathy (BSE) and chronic wasting disease (CWD)) (Table I.1). The transmissible spongiform encephalopathies (TSEs), or prion diseases, are the “poster child” for amyloidogenic diseases that can be transmitted through infectious means and are the primary subject of discussion in this thesis.

The “protein-only” hypothesis and transmissible spongiform encephalopathies:

Prion diseases, or TSEs, are a family of protein misfolding diseases that are responsible for a number of fatal neurodegenerative diseases in several mammalian species, including humans. While most protein misfolding diseases are not infectious, the agents responsible for prion diseases are capable of being transmitted from infected hosts to naïve susceptible hosts [17]. The infectious nature of prion diseases differs from that of all other infectious diseases in that the agent associated with infection lacks nucleic acid, being comprised of only protein. This is known as the protein-only, or “prion” (protein only infectious particle), hypothesis [17]. The central theory of the prion hypothesis focuses on the post-translational conversion from the normal cellular prion protein (PrP^{C}) to the aberrant misfolded disease associated conformer (PrP^{Sc}) (Fig. I.1).

Initial evidence for the protein-only infectious nature of TSE agents came from studies showing that bacterial and viral inactivation treatments, such as UV radiation, did not prevent the

transmission of scrapie, a once thought viral infection that later became known as the archetype TSE [18]. These studies were later confirmed by the purification and separation of the scrapie agent from diseased brain, resulting in the identification of the first known infectious agent to be comprised of only protein [17]. While the exact role the prion protein plays in disease pathogenesis has not been determined, the prion protein is essential in the development of TSE disease [19; 20]—as it has been found that mice devoid of the *PRNP* gene (encoding the prion protein) do not develop TSE disease [21-24].

Prions diseases are known to occur in various mammalian species including: scrapie in sheep [25], bovine spongiform encephalopathy (BSE) in cattle [26], transmissible mink encephalopathy (TME) in hamsters [27; 28], chronic wasting disease (CWD) in cervids (deer, elk, and moose) [29; 30], as well as Fatal Familial Insomnia (FFI), Gerstmann–Sträussler–Scheinker syndrome (GSS), Kuru, and CJD in humans [31-33]. In addition to affecting various species, TSEs can be transmitted to new hosts through numerous routes of exposure—such as intracranial [34; 35], intraperitoneal [36], oral [37-41], intravenous [42-44], aerosol [45; 46], and inadvertent medical [47] exposures—to PrP^{Sc} located in tissues [40; 48-50] and bodily fluids [25; 28; 39; 51-59]. Furthermore, it has been demonstrated that prions can be transmitted in association with contaminated soils/fomites without the need for direct animal-to-animal contact [60-62]. While prion diseases are the only naturally infectious amyloidogenic diseases, other human neurodegenerative diseases, such as AD and PD, have demonstrated prion-like propagation and transmission in experimental settings [33; 63].

PrP^C and PrP^{Sc} distribution:

PrP^C is expressed in tissues throughout the body with the highest expression within neurons and glial cells of the central nervous system (CNS) [22; 64; 65]; however, cells within the lymphoreticular system (LRS) also express high levels of PrP^C [22; 66-70]. PrP^C expression within LRS tissues is of particular importance as studies have revealed the LRS as the earliest site of PrP^{Sc} deposition and replication following peripheral (e.g. IP, oral, or aerosol) inoculation [36; 41; 71-76]. Furthermore, prions are thought to enter the LRS after first crossing the intestinal or nasal epithelial barriers following oral or aerosol inoculation, respectively. In addition to PrP^{Sc} infection of LRS tissues, infectivity has been detected in other non-nervous tissues including skeletal muscle [77-80], cardiac muscle [30; 81], adrenal gland [82], pancreas [82], kidney [83], and liver [72].

While most prion diseases demonstrate systemic involvement, the level of LRS association varies between TSEs. BSE is known to infect numerous tissues outside the central nervous system, including the gastrointestinal (GI) tract, but has been found to have minimal LRS association [84]. Sporadic CJD, while similar to BSE in demonstrating little involvement in extraneural tissues, has been detected in both skeletal muscle and spleen from infected individuals [79; 85]. In contrast to sCJD, the variant strain of CJD (vCJD)—thought to have entered the human population from the consumption of BSE-contaminated beef—has prominent extraneural involvement and a marked tropism for lymphoid organs and tissues [31; 79; 86]. Similar to vCJD, both scrapie and TME have high extraneural involvement—particularly in the LRS—but, CWD stands out for its extensive PrP^{Sc} deposition outside of the CNS [27; 29; 71; 87; 88]. Together scrapie, CWD, TME, and vCJD are known to have the most systemic involvement of all the natural (non-experimental) TSEs.

Chronic wasting disease and transmissible mink encephalopathy:

Chronic wasting disease (CWD) is the only known TSE to occur in a free-ranging wildlife population, naturally infecting elk, moose, and various deer species [89]. First recognized in 1967 in captive mule deer at Colorado State University (Fort Collins, Colorado), the geographical distribution of CWD has expanded across North America, now detected in 23 U.S. states, 2 Canadian provinces, and in South Korea (as of the time of this writing) [90]. The origin of CWD is unknown, as well as how it has efficiently spread throughout North America (some regions have reported a prevalence of 20-30%) [91] and to Korea by importation of CWD-infected elk from North America [92]. CWD has efficient transmission dynamics, likely contributing to its infection of free-ranging and captive cervid populations. Contributing factors to the efficient transmission of CWD may be from direct contact with bodily fluids (blood, saliva, urine, and feces) [39; 61; 93; 94] or through contact with contaminated soils and fomites in the environment [58; 60-62; 91].

Natural transmissible mink encephalopathy (TME) is a TSE of farmed mink and has been detected in Finland, Germany, Canada, the U.S., and the former Soviet Union [95; 96]. Though the origin of TME remains unknown, it is suspected that initial infections were due to ingestion of mink feed scrapie- or BSE-contaminated material [96]. Two distinct strains of TME (Hyper “HY” or Drowsy “DY”) are used in hamster animal models to study the TSE pathogenesis and prion strain interference [96].

CWD and TME recapitulate characteristics of many other TSEs, including human vCJD [29; 96], and therefore provide invaluable insight into the mechanisms of disease transmission and pathogenesis.

CWD and TME pathogenesis:

Previous bioassay studies have established the presence of a protracted subclinical phase of disease for all prion diseases with clinical disease developing months to years after initial TSE exposure, depending on the disease [31; 96]. Cervids infected with CWD develop clinical disease between 15 and 48 months after initial exposure, progressing to terminal clinical disease 2 weeks to 8 months after the initial onset of clinical signs (progressive emaciation, ataxia, polydipsia accompanied by excessive salivation, polyuria, and behavioral changes) [91; 96]. The incubation period for TME-infected mink ranges from 6 to 12 months post exposure, with affected hosts succumbing to terminal disease between 2 to 8 weeks post clinical onset (behavioral changes and ataxia, with the addition tremors, declined coordination, and convulsions) [96].

The evolving field of prion detection:

Conventional prion detection has relied upon immunohistochemistry (IHC), western blot analysis, and bioassay. All three methodologies have proven to provide challenges in the detection of blood-borne prions. Western blot analysis of blood or tissues with large volumes of blood has proven difficult due to the presence of compounds that inhibit western blot detection. IHC is ineffective for blood analysis as it is tissue based. While bioassay in native host and rodent models possesses the sensitivity necessary to detect hematogenous prions, they are unrealistic diagnostic tools due to their cost and time requirements.

The development of highly sensitive *in vitro* PrP conversion or amplification assays has enhanced our ability to detect prions in tissues and bodily fluids, and has significantly reduced detection time and expense compared to bioassay. Serial protein misfolding cyclic amplification

(sPMCA), one of the initial amyloid amplification assays, provided the first *in vitro* detection of hematogenous prions [97-99]. Similar to the polymerase chain reaction, sPMCA functions through continuous cycles of fragmentation and elongation. Prion-infected tissue or blood (containing PrP^{Sc}) is combined with brain homogenate expressing PrP^C from uninfected animals. During the elongation steps the PrP^C in the brain homogenate provides the necessary substrate for amplification of PrP^{Sc}. The samples then undergo sonication to fragment the PrP^{Sc} fibrils into multiple smaller pieces upon which further amyloid formation can occur.

While able to detect blood-borne prions halfway through infection and after, sPMCA has insufficient sensitivity to detect the very small quantities of prions present early in infection. This is thought to be due to inhibitory compounds present in whole blood and its reliance upon protease digestion prior to western blot immunoassay analysis. To overcome these limits sPMCA studies have focused on the use of buffy coat fractions of whole blood instead of pure whole blood, with limited success [97; 98]. Given that infectious prions have been detected in the red blood cell fraction of whole blood, analyzing only part of the whole blood may contribute to decreased assay sensitivity. However, the low concentration of prions in blood and the presence of assay inhibitors in whole blood may still pose a problem when trying to detect prions in whole blood samples. Thus, methods to remove these inhibitors and/or concentrate PrP^{Sc} from other proteins present in whole blood are warranted.

The next generation of PrP^C-converting assay, real-time quaking-induced conversion (RT-QuIC) [100-102], has provided many advances. Similar to sPMCA, RT-QuIC relies upon the seeded conversion of a PrP^C rich substrate into the misfolded isoform. Where sPMCA uses normal brain homogenate expressing PrP^C for this conversion, RT-QuIC employs the use of recombinant prion protein (rPrP) as the templating substrate. PrP^{Sc} in the seeding material

initiates the rPrP to undergo a conformational change into an amyloid isoform. This growing amyloid fibril is detected by the intercalation of a fluorescence marker, thioflavin T (ThT), within the structured β -sheets of the amyloid [103]. Both sPMCA and RT-QuIC offer the potential for enhanced ante-mortem hematogenous prion detection. RT-QuIC provides the added benefit of a non-mouse source substrate incorporating real-time fluorescence readout. As with all *in vitro* assays, RT-QuIC has its own limitations, including the generation of the rPrP substrate. Studies published as part of this thesis describe the optimization of the RT-QuIC assay for use with whole blood, the first *in vitro* temporal status of conversion-competent blood borne-prions from minutes post TSE exposure through terminal TSE disease, and the first demonstration of a primary and secondary prionemia (Elder et al., 2015 Submitted for publication)[104].

A history of hematogenous prions:

Infectious prions are associated with various tissues and bodily fluids harvested from TSE-infected hosts. An understanding of the true infectious nature of bodily fluids has been shown by bioassay of blood [25; 43; 44; 51; 52; 54; 94; 105; 106], saliva [51; 107], urine [39; 107], and feces [39; 58]. Further confirmation that these fluids contain aggregated misfolded prions has been supported by various *in vitro* detection methodologies [53; 93; 97; 98; 104; 108]. Hematogenous prions have been detected in mammalian hosts infected with BSE [109], scrapie [25; 54], TME [104], CWD [51; 94], sCJD and vCJD [105; 109; 110].

The study of blood-borne prion transmission has been ongoing since the 1960's, making it one of the longest projects in the field of prion research. The first evidence that blood from TSE-infected animals harbor infectious material came from early bioassays in the 1960's and 70's when it was demonstrated that blood collected from scrapie-infected sheep could infect

goats and a mouse model [111-113]. Despite incomplete attack rates, detection of scrapie in LRS tissues harvested from these studies indicated that infectious prions were carried with blood from scrapie-infected sheep. More recent studies conducted by Hunter and Houston found that sufficient infectious prions are present in a transfusion of 500 ml of whole blood or buffy coat (500ml equivalent in cells) from scrapie-infected sheep to transmit disease [25; 54].

Additionally, Mathiason et al. demonstrated CWD transmissibility to cervids via intravenous inoculation of 250 ml whole blood harvested from subclinical CWD-infected cervid [51].

Transfusion transmission of infectious prions has occurred in humans. In 1996 a variant strain of CJD (vCJD) was described for the first time. Variant CJD has since been linked—via biochemical and strain typing analysis—to the transmission of BSE to humans through the consumption of BSE-contaminated meat. To date, there have been 229 confirmed cases of vCJD worldwide, of which, 4 have acquired the disease through contaminated blood transfusion [44; 114-117]. In these occasions, donated blood had been transfused to CJD naïve individuals from donors who were unknowingly infected with vCJD at the time of donation, but later developed clinical disease. Following these incidences of transmission via blood from subclinical donors the UK has employed mandatory leukocyte-reduction of donated blood products in an attempt to reduce the risk of transmission. Unfortunately, the methods of reduction have been shown to be incapable of removing 100% of infectious prions from blood [118-120]. Additionally, based on a recent retrospective analysis of appendix and tonsil tissues from deceased patients, it is currently thought that vCJD may be present in the subclinical disease state in as many as 1/1,250 persons in the United Kingdom [86; 121; 122]. Thus, concern exists that vCJD may still be present in the human population with the capability of transmitting covertly through blood transfusion or iatrogenic transmission.

Aside from transfusion studies aiming to determine if blood harbors infectious prions, hematogenous prion research has also focused on: finding the minimum infectious dose, determining what blood components carry infectivity, developing a blood-based diagnostic tool for prion detection, and determining if prionemia onset begins in the subclinical phase of disease.

While a true minimum infectious dose has yet to be found, recent studies have demonstrated that transfusion of 200 µl of scrapie-infected whole blood was sufficient to transmit infection to naïve sheep [52]. These results indicate that blood may contain more infectivity than previously thought. Other studies have focused on establishing prion association in blood—cellular, acellular, or both. B cells have long been implicated in prion infection and LRS involvement, and are known to harbor infectious prions capable of transmitting disease [36; 85; 94; 123-126]. Other immune cells, including follicular dendritic cells (FDCs) and monocytes/macrophages, have been associated with PrP^{Sc} deposition [36; 74; 123]. Virtually all components of blood have been associated with infectious prions including white blood cells [52; 127], plasma [105; 128], platelets [94; 127; 129], and erythrocytes [105]. The discovery that whole blood carries prion infectivity has led to precautionary leukodepletion [119]. However, the presence of infectivity in erythrocytes may render the practice of leukodepletion unsatisfactory in eliminating the concern for hematogenous TSE dissemination. It has been established for several TSEs (scrapie, CWD, TME, sCJD, and vCJD) that infectious prions are present during the long asymptomatic, or subclinical, phase of disease lasting several months to years [98; 104; 109; 130]. Determining the biological role of blood in TSE disease pathogenesis may contribute significantly to our overall understanding of TSEs and other protein misfolding diseases impacting all mammals, including humans.

Significance of hematogenous prions in TSE pathogenesis:

Prion-infected individuals can persist in a subclinical carrier state for months or years prior to the development of clinical disease [31; 96]. Infectious prions are known to be present in the blood during this subclinical stage of disease [43; 116]. During this carrier state, blood-borne prions are most certainly trafficked throughout the body and are involved in the infection of extraneural tissues, and possibly the CNS itself.

The mechanism(s) by which prions infiltrate the CNS during natural infection are still unknown. It is suspected that prions enter through the peripheral nervous system (PNS), blood, or both. In the PNS scenario, infectious prions enter the body and are transported directly to regional lymphoid tissue (tonsils, GALT, NALT, etc...) followed by drainage into regional lymph nodes (e.g. retropharyngeal or mesenteric lymph nodes). From regional lymph nodes infectious prions are trafficked in the blood and lymph to non-GALT lymphoid tissues, such as the spleen. Subsequent neuroinvasion may then occur by retrograde transport using the sympathetic and parasympathetic neurons innervating these tissues [73; 74].

A second hypothesis, that neuroinvasion occurs via blood-borne prions, supports prion entry via the circumventricular organs (CVOs), breaching the blood brain barrier [41; 73]. In this scenario, neuroinvasion occurs after infectious prions enter the blood and are trafficked to tissues throughout the body, including lymphoid tissues and the brain [41; 73]. This hypothesis is reinforced by recent findings [131], which revealed that hamsters inoculated intraperitoneally had detectable prions in the brain as early as 2 days post inoculation. Further support comes from observations that PrP^{Sc} accumulation in CVOs occurs irrespective of inoculation route and that deposition occurs first at the CVOs with subsequent invasion of the corresponding parenchymal regions, rather than a random distribution throughout the brain [41; 73; 132].

Studies published as part of this thesis demonstrate that prions can quickly translocate across mucosal barriers and gain entry into the circulatory system where they are detectable throughout the entire course of disease. These results make it plausible that neuroinvasion via hematogenous prions may occur earlier in the disease course than previously considered for all TSEs. The rapid infection of extraneural tissues may result in peripheral amplification throughout the course of disease of prions in these tissues with spillover of prions back into the blood. This process may be vital in the shedding of prions throughout the entirety of disease.

Thesis research:

The overall objective of this work was to determine the temporal status of blood-borne prions in animals infected via various routes of inoculation. The tools developed and used in this thesis research include a sensitive and efficient *in vitro* method to detect amyloid formation (i.e. prions) and longitudinally collected blood samples from TSE-infected animals. We used blood collected throughout the entire course of disease in CWD- and TME-infected animals, as longitudinal blood samples from vCJD-infected hosts are not available. We hypothesized that prions are trafficked in the vascular system of infected individuals early in the subclinical stage of disease, and that they could be detected by highly sensitive conversion assays (RT-QuIC) in whole blood (wbRT-QuIC). We developed optimal detection conditions and treatments for the detection of the small quantities of PrP^{Sc} present in the whole blood of CWD- and TME-infected hosts.

The use of RT-QuIC on whole blood samples provides a highly sensitive (94%) and specific (100%) means of detecting the presence of amplification-competent prions. We hypothesized that prions enter the blood during subclinical infection, regardless of inoculation

route, and that the use of wbRT-QuIC would demonstrate the earliest establishment of prionemia. Analysis by wbRT-QuIC uncovered the temporal status and exposure dynamics of hematogenous prions. We describe for the first time: 1) *in vitro* detection of hematogenous prions present in whole blood collected from longitudinal TSE-exposed cervids and hamsters; 2) prions were able to transverse mucosal barriers to enter the blood as early as 15 minutes post TSE exposure (oral, aerosol, intravenous, and extranasal), and were present throughout the development of terminal TSE disease; and 3) a distinct primary and secondary prionemia—likely representing the inoculum (primary prionemia), a host clearance mechanism, and the generation of *de novo* prions (secondary prionemia) in TSE-infected hosts.

The primary goal of this thesis was to determine the *in vitro* temporal status of hematogenous prions for TSE-exposed hosts with the motivation that our discoveries would provide critical information about the pathogenesis of TSEs and possibly other amyloidogenic diseases.

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CHAPTER 1¹

In Vitro Detection of Prionemia in TSE-infected Cervids and Hamsters

OVERVIEW

Blood-borne transmission of infectious prions during the symptomatic and asymptomatic stages of disease occurs for both human and animal transmissible spongiform encephalopathies (TSEs). The geographical distribution of the cervid TSE, chronic wasting disease (CWD), continues to spread across North America and the prospective number of individuals harboring an asymptomatic infection of human variant Creutzfeldt-Jakob Disease (vCJD) in the United Kingdom has been projected to be ~1 in 3000 residents. Thus, it is important to monitor cervid and human blood products to ensure herd health and human safety. Current methods for detecting blood-associated prions rely primarily upon bioassay in laboratory animals. While bioassay provides high sensitivity and specificity, it requires many months, animals, and it is costly. Here we report modification of the real time quaking-induced conversion (RT-QuIC) assay to detect blood-borne prions in whole blood from prion-infected preclinical white-tailed deer, muntjac deer, and Syrian hamsters, attaining sensitivity of >90% while maintaining 100% specificity. Our results indicate that RT-QuIC methodology as modified can provide consistent

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and reliable detection of blood-borne prions in preclinical and symptomatic stages of two animal TSEs, offering promise for prionemia detection in other species, including humans.

INTRODUCTION

The hematogenous spread of prions in transmissible spongiform encephalopathy (TSE)-infected animals has long been hypothesized [1-3], but evidence for the presence of prions in non-nervous/lymphoid tissues and blood was not available for several decades [4-8]. Later studies have provided unequivocal proof of efficient TSE blood-borne infectivity [9-14]. The knowledge that prions traffic throughout the body in blood has important implications for both human and animal health.

Variant Creutzfeldt-Jakob disease (vCJD) emerged following the bovine spongiform encephalopathy (BSE) epidemic in the United Kingdom in the 1980s and 90s. Biochemical and strain typing analysis have provided evidence indicating that vCJD originated from human exposure to BSE contaminated material. To date, 227 cases of vCJD have been diagnosed worldwide [15], four of which have been transmitted by non-leukodepleted blood transfusion [16-20]. While leukocyte reduction has been implemented to filter prions and prion carrying cells from blood products, these filtration methods are unable to remove 100% of TSE infectivity [8; 21; 22]. In addition, recent reports have revealed that 1/1,250 to 1/3,500 persons in the United Kingdom may be asymptomatic carriers of vCJD as a result of the BSE epidemic [23]. Thus, concern exists that a secondary outbreak of vCJD may ensue involving blood-borne prion transmission originating from individuals unknowingly carrying a subclinical prion infection.

Here we address the need for an *in vitro* assay with the ability to detect the prion disease-associated isoform of prion protein (PrP^D) present in whole blood.

Several animal TSEs, including chronic wasting disease (CWD) of deer and elk [13; 24] and hamster-adapted transmissible mink encephalopathy (TME) [25; 26] exhibit a hematogenous phase of infection, thus providing excellent TSE models for the development of an ante-mortem blood-borne PrP^D detection assay.

While traditional assays, such as Western blot and immunohistochemistry (IHC), are effective for detecting large quantities of prions present in nervous and lymphoid tissues, they do not have the ability to detect the minute quantities of prions thought to be present in bodily fluids or peripheral tissues early in infection. Rodent bioassays have the necessary sensitivity and specificity to detect hematogenous prions, but they are not realistic as rapid and cost-effective diagnostic tools. *In vitro* prion detection was advanced with the advent of serial protein misfolding cyclic amplification (sPMCA) [25; 27]. sPMCA has been optimized for the detection of prions in blood [26] and requires less time than bioassay, but its use has been hampered by a lack of consistent sensitivity and a dependence on protease digestion prior to immunoassay readout. In contrast, the real-time quaking-induced conversion (RT-QuIC) assay [28-30] relies upon the seeded conversion of recombinant prion protein (rPrP) to PrP^D and subsequent binding of the fluorescence marker, thioflavin T (ThT), to the resulting amyloid isoforms [31]. This process offers enhanced ante-mortem prion detection and real-time fluorescence readout [30].

We undertook this project to determine if adaptations applied to RT-QuIC could provide a fast, sensitive and consistent assay for the detection of blood-borne prions.

MATERIALS AND METHODS

Ethics Statement:

All animals were handled in strict accordance with guidelines for animal care and use provided by the United States Department of Agriculture (USDA), National Institutes of Health (NIH) and the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), and all animal work was approved by Colorado State University Institutional Animal Care and Use Committee (IACUC) Institutional Animal Care and Use Committee (IACUC) (approval numbers 02-151A, 08-175A and 11-2615A). All procedures involving hamsters were preapproved by the Creighton University Institutional Animal Care and Use Committee and were in compliance with the *Guide for the Care and Use of Laboratory Animals*.

Cervid inoculations:

Cervid whole blood was procured from historical and contemporary white-tailed and muntjac deer studies conducted at CSU (Table 1). Prior to inoculation, cervids were anesthetized with a mixture of ketamine and medetomidine. In brief, naïve 1-2 year old white-tailed deer (*Odocoileus virginianus*) were inoculated with CWD-positive material as follows: 1) 1.0 g of brain in a 10% brain homogenate (10 ml) administered intracranially [13]; 2) 250 ml fresh/frozen whole blood administered intravenously/intraperitoneally, respectively [13]; 3) 1.0 g of brain in a 10% brain homogenate administered orally; or 4) 2 ml of a 5% (wt/vol) brain homogenate aerosol-administered [32]. Negative control white-tailed deer were exposed to sham inoculum as described above. Naïve 1-2 year old muntjac deer (*Muntiacus reevesi*) were inoculated with 1.0 g

total brain in a 10% brain homogenate administered orally/subcutaneously [33]. Negative control muntjac deer received sham inoculum as described above.

Table 1.1 Cervid blood donor inoculations, clinical status, and assay results

Animal #	Inoculum	Route of Inoculation	Sample collection date	Disease Status	Western Blot Status (Obex)	IHC Status	Positive QuIC Replicates
1 (WTD)	2 ml 5% CWD+ brain homogenate	Aerosol	23 MPI	Clinical	+	+ ^B	8/8
2 (WTD)	2 ml 5% CWD+ brain homogenate	Aerosol	22 MPI	Clinical	+	+ ^B	8/8
3 (WTD)	2 ml 5% CWD+ brain homogenate	Aerosol	22 MPI	Clinical	+	+ ^B	7/8
4 (WTD)	2 ml 5% CWD+ brain homogenate	Aerosol	23 MPI	Clinical	+	+ ^B	8/8
5 (WTD)	2 ml 5% CWD+ brain homogenate	Aerosol	19 MPI Termination	Pre-clinical	+	+ ^B	8/8
6 (WTD)	2 ml 5% CWD+ brain homogenate	Aerosol	16.5 MPI Termination	Clinical	+	+ ^B	8/8
7 (WTD)	1.0 g 10% CWD+ brain homogenate	PO	24.5 MPI	Pre-clinical	NA	+ ^B	7/8
8 (WTD)	1.0 g 10% CWD+ brain homogenate	PO	22 MPI Termination	Pre-clinical	+	+ ^B	8/8
9 (WTD)	1.0 g 10% CWD+ brain homogenate	PO	22 MPI	Pre-clinical	-	- ^B	7/8

	homogenate						
10 (WTD)	1.0 g 10% CWD+ brain homogenate	PO	22 MPI Termination	Clinical	+	+ ^B	8/8
11 (WTD)	1.0 g 10% CWD+ brain homogenate	PO	16 MPI Termination	Pre- clinical	+	+ ^B	8/8
12 (WTD)	1.0 g 10% CWD+ brain homogenate	PO	18 MPI Termination	Clinical	+	+ ^B	7/8
13 (WTD)	1.0 g 10% CWD+ brain homogenate	PO	22 MPI Termination	Clinical	+	+ ^B	8/8
14 (WTD)	1.0 g 10% CWD+ brain homogenate	PO	16 MPI Termination	Pre- clinical	+	+ ^B	7/8
15 (WTD)	2.0 g 10% CWD+ brain homogenate	IC	12 MPI Termination	Clinical	+	+ ^{B,O}	7/8
16 (WTD)	250 ml CWD+ whole blood	IV	12 MPI	Clinical	+	+ ^{B,O}	8/8
17 (MJ)	1.0 g 10% CWD+ brain homogenate	PO/SQ	6.5 MPI Termination	Pre- clinical *	-	+ ^O	8/8
18 (MJ)	1.0 g 10% CWD+ brain homogenate	PO/SQ	6.5 MPI Termination	Pre- clinical *	-	+ ^O	6/8
19 (MJ)	1.0 g 10% CWD+ brain homogenate	PO/SQ	26 MPI Termination	Clinical	+	+ ^{B,O}	8/8
20 (MJ)	1.0 g 10% CWD+ brain homogenate	PO/SQ	23 MPI Termination	Clinical	+	+ ^{B,O}	8/8
21 (MJ)	1.0 g 10%	PO/SQ	22 MPI	Clinical	+	+ ^{B,O}	8/8

CWD+ brain homogenate		Termination					
22 (MJ)	1.0 g 10% CWD+ brain homogenate	PO/SQ	24 MPI Termination	Clinical	+	+ ^{B,O}	4/8
23 (WTD)	2 ml sham homogenate	Aerosol	19 MPI	NA	-	- ^B	0/8
24 (WTD)	2 ml sham homogenate	Aerosol	23 MPI	NA	-	- ^B	0/8
25 (WTD)	2 ml sham homogenate	Aerosol	22 MPI	NA	-	- ^B	0/8
26 (WTD)	Uninfected urine/feces	PO	20 MPI	NA	-	- ^{B,O}	0/8
27 (WTD)	Uninfected urine/feces	PO	20 MPI	NA	-	- ^{B,O}	0/8
28 (MJ)	1.0 g sham homogenate	PO/SQ	13 MPI	NA	-	- ^O	0/8
29 (MJ)	1.0 g sham homogenate	PO/SQ	23 MPI	NA	-	- ^O	0/8
30 (MJ)	Uninoculate d	NA	NA	NA	-	- ^O	0/8
31 (MJ)	Uninoculate d	NA	NA	NA	-	- ^O	0/8
32 (MJ)	Uninoculate d	NA	NA	NA	-	- ^O	0/8
33 (MJ)	Uninoculate d	NA	NA	NA	-	- ^O	0/8

WTD = White-tailed deer; MJ = Muntjac deer; NA = Not available; - = PrP^D was not detected in the sample; + = PrP^D was detected in the sample; MPI= Months post inoculation; ^B = Biopsy of tonsil and recto-anal mucosa associated lymphoid tissue; ^O = Obex; * = Less than halfway to clinical disease

Hamster inoculation:

Male 10-11 week old Syrian hamsters (Harlan Sprague Dawley, Indianapolis, IN) were used in these studies. Extranasal (e.n.) inoculations using a 10% w/v brain homogenate containing 10^{6.8} intracerebral 50% lethal doses per ml of the HY TME agent or a sham

homogenate were performed as previously described [34]. Hamsters receiving e.n. inoculations were briefly anesthetized with isoflurane (Webster Veterinary), placed in a supine position and 5 µl of brain homogenate was placed just inferior to each nostril (10 µl total volume). Brain homogenate was immediately inhaled into the nasal cavity, as hamsters are obligate nose breathers.

Blood and tissue collection from cervids:

Whole blood (10 ml/cervid/anticoagulant) was collected from n=22 CWD-inoculated cervids following anesthetization with ketamine and medetomidine — six in various stages of disease presentation and 16 at termination— and from 11 negative control sham-inoculated cervids (Table 1). All blood samples were preserved in one of three anticoagulants: 1) 14% anticoagulant *citrate phosphate dextrose adenine (CPDA)*, 2) 15% ethylenediaminetetraacetic acid (EDTA), or 3) 200 units/ml heparin, before being placed in 1 ml aliquots and frozen at -80°C. At termination cervids were euthanized with beuthanasia-D solution. Brain (medulla oblongata) collected from each terminal white-tailed deer and muntjac deer was frozen at -80°C or fixed in 10% neutral buffered formalin or paraformaldehyde-lysine-periodate (PLP) and stored in 60% ethanol prior to processing.

Blood and tissue collection from hamsters:

At selected time points post-infection, three infected and one mock-infected hamster were anesthetized with isoflurane and blood was collected via cardiac puncture into heparin blood tubes for preservation at -80°C (Table 2). The animals were then transcardially perfused with 50 ml of 0.01 M Dulbecco's phosphate buffered saline followed by 75 ml of McLean's PLP

fixative. Brain and brainstem were immediately removed and placed in PLP for 5-7 hours at room temperature prior to paraffin processing and embedding.

Table 1.2 Hamster blood donor inoculations, clinical status, and assay results

Animal #	Inoculum	Route of inoculation	Disease Status	Sample collection date	IHC Status	Positive QuIC Replicates
34	10 µl 10% HY TME brain homogenate	Extranasal	Pre-clinical*	8 WPI	ND	8/8
35	10 µl 10% HY TME brain homogenate	Extranasal	Pre-clinical*	8 WPI	ND	8/8
36	10 µl 10% HY TME brain homogenate	Extranasal	Pre-clinical*	8 WPI	ND	5/8
37	10 µl 10% HY TME brain homogenate	Extranasal	Pre-clinical*	10 WPI	ND	8/8
38	10 µl 10% HY TME brain homogenate	Extranasal	Pre-clinical*	10 WPI	ND	5/8
39	10 µl 10% HY TME brain homogenate	Extranasal	Pre-clinical*	10 WPI	ND	8/8
40	10 µl 10% HY TME brain homogenate	Extranasal	Pre-clinical	12 WPI	-	8/8
41	10 µl 10% HY TME brain homogenate	Extranasal	Pre-clinical	12 WPI	-	8/8
42	10 µl 10% HY TME brain homogenate	Extranasal	Pre-clinical	12 WPI	-	8/8

43	10 µl 10% HY TME brain homogenate	Extranasal	Pre-clinical	14 WPI	-	8/8
44	10 µl 10% HY TME brain homogenate	Extranasal	Pre-clinical	14 WPI	+	8/8
45	10 µl 10% HY TME brain homogenate	Extranasal	Pre-clinical	14 WPI	+	8/8
46	10 µl 10% HY TME brain homogenate	Extranasal	Pre-clinical	16 WPI	+	8/8
47	10 µl 10% HY TME brain homogenate	Extranasal	Pre-clinical	16 WPI	+	7/8
48	10 µl 10% HY TME brain homogenate	Extranasal	Pre-clinical	16 WPI	+	8/8
49	10 µl 10% HY TME brain homogenate	Extranasal	Pre-clinical	18 WPI	ND	8/8
50	10 µl 10% HY TME brain homogenate	Extranasal	Pre-clinical	18 WPI	ND	7/8
51	10 µl 10% HY TME brain homogenate	Extranasal	Pre-clinical	18 WPI	ND	8/8
52	10 µl 10% HY TME brain homogenate	Extranasal	Clinical	20 WPI	ND	8/8
53	10 µl 10% HY TME brain homogenate	Extranasal	Clinical	20 WPI	ND	7/8
54	10 µl 10% HY TME	Extranasal	Clinical	20 WPI	ND	8/8

	brain homogenate					
55	10 µl 10% sham homogenate	Extranasal	NA	8 WPI	-	0/8
56	10 µl 10% sham homogenate	Extranasal	NA	10 WPI	-	0/8
57	10 µl 10% sham homogenate	Extranasal	NA	12 WPI	-	0/8
58	10 µl 10% sham homogenate	Extranasal	NA	14 WPI	-	0/8
59	10 µl 10% sham homogenate	Extranasal	NA	16 WPI	-	0/8
60	10 µl 10% sham homogenate	Extranasal	NA	18 WPI	-	0/8
61	10 µl 10% sham homogenate	Extranasal	NA	20 WPI	-	0/8

WPI = weeks post inoculation; NA = Not available; ND = Not done; - = PrP^D was not detected; + = PrP^D was detected in the sample; *= Less than/equal to the halfway point to clinical disease

Brain tissue homogenization:

Ten percent (10%) brain tissue homogenates were prepared from the obex region of the medulla oblongata by homogenizing 0.05 g brain tissue in 0.5 ml homogenate buffer (1X PBS + 0.1% Triton-X 100 [Sigma-Aldrich]). Samples were homogenized using 0.5 mm diameter zirconium oxide beads and a Bullet Blender (Next Advance) for 5 minutes at a speed setting of 10. Homogenates were stored at -80°C in 20 µl aliquots.

Whole blood freeze-thaw and homogenization process:

One milliliter (1 ml) aliquots of whole blood were frozen at -80°C for 30 minutes and subsequently thawed at 22°C for 60 minutes. This process was repeated four times. Samples

were then homogenized using 0.5 mm diameter zirconium oxide beads and a Bullet Blender (Next Advance) for 5 minutes at top speed.

Sodium phosphotungstic acid (NaPTA) precipitation:

Sodium phosphotungstic acid (NaPTA) precipitation of prions, as first described by Wadsworth et al. [35], was used to concentrate proteins (including PrP) present in whole blood samples. Frozen whole blood homogenates were thawed and centrifuged at 2000 rpm for one minute to remove cellular debris. Five hundred microliters (500 μ l) of supernatant were mixed with an equal volume of 4% sarkosyl in 1X phosphate buffered saline (PBS) and incubated for 30 minutes at 37°C with constant agitation. Samples were then adjusted to contain a final concentration of 50 U/ml of benzonase (Sigma-Aldrich) and incubated at 37°C for another 30 minutes with constant agitation. A solution of 4% (w/v) phosphotungstic acid (Sigma-Aldrich) and 170 mM magnesium chloride, adjusted to pH 7.4 with NaOH, was added to the sample for a final concentration of 0.3% (w/v) NaPTA and agitated at 37°C for 30 minutes. Samples were then centrifuged for 30 minutes at 14,000 rpm and the pellet was resuspended in 50 μ l 0.1% (v/v) sarkosyl.

Recombinant protein preparation:

Recombinant protein was expressed and purified as previously described [36; 37]. Truncated recombinant Syrian hamster PrP (SHrPrP 90-231; received from the Caughey laboratory) expressed by Rosetta strain *Escherichia coli* was inoculated into 1 liter of LB containing Auto Induction™ supplements (EMD Biosciences). Cultures were allowed to grow overnight until harvest when an OD (600nm) of \sim 3 was reached. Cells were lysed using Bug

Buster™ and Lysonase™ (EMD Biosciences). Inclusion bodies (IB) were isolated by centrifugation at 15,000xg and were solubilized in 8 M guanidine hydrochloride in Tris-phosphate buffer (100 mM NaPO₄ and 10 mM Tris pH 8.0). The protein solution obtained was bound to Super Flow Ni-NTA resin (Qiagen) pre-equilibrated with denaturing buffer (6.0 M GuHCl Tris-phosphate) at room temperature with agitation for 45 minutes and added to a XK FPLC column (GE). SHrPrP was refolded on the column with refolding Tris-phosphate buffer at 0.75 ml/min for 340 ml, then eluted with 0.5 M imidazole Tris-phosphate pH 5.5 at 2.0 ml/min for a total of 100 ml. Eluted fractions were collected and dialyzed in two changes of 4.0 liters dialysis buffer (20 mM NaPO₄ pH 5.5). Following dialysis, purified protein was adjusted to 0.6 mg/ml, flash frozen in 1 ml aliquots, and stored at -80°C.

Real-time quaking induced conversion (RT-QuIC) assay:

Real-time quaking induced conversion (RT-QuIC), first described by Atarashi et al. [28], Wilham et al. [30], and Orru et al. [29], was used for the conversion of small quantities of prions present in the blood of TSE-infected animals. Positive assay controls and samples consisted of serial dilutions of a 10% homogenate of CWD or TME-infected brain (10^{-3} - 10^{-9}) and NaPTA precipitated blood from infected animals (10^0 - 10^{-6}), respectively. Negative assay controls and samples were comprised of serial dilutions of a 10% homogenate of uninfected brain (10^{-3} - 10^{-9}) and NaPTA precipitated blood from uninfected animals (10^0 - 10^{-6}), respectively. RT-QuIC reactions were set up in 96-well clear bottom optic plates (Nalgene Nunc) and consisted of 98 μ l RT-QuIC Buffer (final concentrations of 1X PBS, 1 mM EDTA, 10 μ M Thioflavin T (ThT), 100-200 mM NaCl buffer, and 0.1 mg/ml recombinant Syrian hamster PrP^C substrate) and 2 μ l sample. Blood samples that were placed into the whole blood optimized RT-QuIC (WBO RT-

QuIC) assay were 2 μ l of serial dilutions made from concentrated material of 500 μ l. Once reactions were set up in each well, plates were placed in a BMG Fluostar fluorescence plate reader with settings of 42°C for 60 hours with cycles consisting of 1 minute shake, 1 minute rest and ThT fluorescence measurements were taken every 15 minutes. Data were processed using Microsoft Excel (Microsoft Inc.) prior to graph production with Prism 6 (GraphPad Prism).

Cervid immunohistochemistry:

Samples were processed and analyzed as previously described by Nalls et al. [33]. In brief, fixed tissues were treated with formic acid, embedded in paraffin, cut, and placed on positively charged slides. Deparaffinized, rehydrated and PK digested (20 mg/ml) tissues underwent epitope retrieval and were probed with primary antibody BAR224 (Cayman Chemical) and secondary anti-mouse HRP labeled polymer (Dako) prior to counterstain and reading by light microscopy

Hamster immunohistochemistry:

Immunohistochemistry was performed to detect PrP^D as previously described [34]. In brief, deparaffinized, formic acid treated tissue sections were processed for antigen retrieval. Endogenous peroxidase and non-specific staining were blocked in H₂O₂ in methanol and normal horse serum. The sections were probed with monoclonal anti-PrP antibody 3F4 followed by secondary biotinylated horse anti-mouse immunoglobulin G conjugate prior to detection with ABC solution (Elite kit; Vector Laboratories). The sections were counterstained with hematoxylin and read by light microscopy

Western blotting:

Western blotting performed as previously described [38] with the following modifications: tissue homogenates were mixed with proteinase K (PK) (Invitrogen) to a final concentration of 50 µg/ml and incubated at 37°C for 30 minutes, followed by incubation at 45°C for 10 minutes with constant agitation. Samples were size fractionated on a NuPAGE 10% Bis-Tris gel (Novex) in 1X MOPS buffer at 100 volts for 2.5 hours, transferred to a polyvinylidene fluoride (PVDF) membrane for 7 minutes using the Trans-blot Turbo transfer system (Biorad). Post-transfer, the PVDF membrane was loaded onto a wetted SNAP i.d. holder (Millipore) and placed in the SNAP i.d. vacuum filtration system (Millipore). The PVDF membrane was blocked for 10 minutes with Blocking Buffer (Blocker casein in TBS [Thermo Scientific] with 0.1% Tween 20), and incubated for 10 minutes with 0.2 µg/ml primary antibody BAR224 (Cayman Chemical) -HRP conjugated antibody. The membrane was washed with TBST and developed using ECL Plus enhanced chemiluminescence Western blotting detection reagents (Invitrogen) and imaged on a Luminescence image analyzer LAS 3000 (Fujifilm).

Mouse titration bioassay:

All animals were handled in strict accordance with guidelines for animal care and use provided by the United States Department of Agriculture (USDA), National Institutes of Health (NIH) and the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), and all animal work was approved by Colorado State University Institutional Animal Care and Use Committee (IACUC). Seven cohorts of TgCerPrP mice (n=9) were inoculated with 30 µl of a CWD-infected cervid brain homogenate intracranially. Each cohort received a different concentration of inoculum ranging from 10% (w/v) to 0.00001%

(w/v). Negative control mice were inoculated with sham material. Mice were subsequently observed and terminated upon onset of clinical disease. All mice were analyzed for the presence of PrP^D by Western blot and immunohistochemistry.

Calculations:

RT-QuIC assay sensitivity was determined by analyzing the number of replicates demonstrating positivity compared to the total number of replicates run (# of positive replicates/total replicates analyzed). Separate calculations were performed for animals with clinical disease status and animals with subclinical disease status.

PrP^D concentration in blood was determined by comparison of the time to positivity for whole blood and brain samples. 2 μ l of a 1/100 dilution (blood seed= 2×10^{-2} μ l) of NaPTA treated whole blood was compared against a dilutional series of brain samples. A 10 % brain homogenate was used and serially diluted by 10-fold dilutions to 10^{-8} . 2 μ l of 10^{-5} - 10^{-8} dilutions were seeded into the RT-QuIC assay. Calculations for 10^{-6} dilution of brain in RT-QuIC are used as an example: $0.1 \text{ g/ml diluted } 10^{-6} = (10^{-10} \text{ g}/\mu\text{l})(2 \mu\text{l}) = 2 \times 10^{-10} \text{ g} = 0.2 \text{ ng}$.

LD₅₀ and SD₅₀ were calculated using the Reed-Muench method [39].

RESULTS

RT-QuIC analysis of whole blood collected in various anticoagulants:

To determine the influence of common blood preservation reagents in *in vitro* PrP^D detection assays, we compared the ability of RT-QuIC to amplify CWD prions in fresh cervid whole blood preserved in CPDA (citrate phosphate dextrose adenine), EDTA

(ethylenediaminetetraacetic acid), or heparin. Samples were run in serial dilutions (10^0 - 10^{-6}) in the RT-QuIC assay to determine the optimal dilution for PrP^D detection. While RT-QuIC PrP^C-converting activity was observed in heparin-preserved blood from CWD-infected deer (1/2 replicates in one dilution; 10^{-5}), PrP^C-converting activity was not detected in CPDA or EDTA preserved blood from the same animal or any blood collected from sham-inoculated deer (Figure 1). This experiment was repeated six times with fresh whole blood with similar results witnessed each time. All subsequent RT-QuIC analyses were conducted on whole blood harvested in heparin.

RT-QuIC analysis of fresh versus frozen whole blood:

To determine if historical blood samples were adequately preserved to initiate PrP^C-converting activity in RT-QuIC, whole blood was collected from contemporary naïve and CWD-infected white-tailed deer and compared as fresh versus frozen samples. Samples were processed in various dilutions ranging from undiluted to 10^{-6} to determine the optimal dilution for PrP^D detection using frozen whole blood in the RT-QuIC assay. While PrP^C-converting activity was detected in fresh whole blood, blood that had been processed through the freeze-thaw procedure yielded higher and more consistent detection of PrP^C-converting activity (2/2 replicates in the 10^{-3} , 10^{-4} and 10^{-6} dilutions; 1/2 replicates in the 10^{-5} dilution) (Figure 2). PrP^C-converting activity was not observed in wells containing only substrate or naïve cervid blood. To determine if the results observed in the anticoagulant study were due solely to the use of fresh blood, the experiments were repeated on frozen blood collected in all three anticoagulants. Results revealed identical outcomes for both CPDA and EDTA blood while showing an increased sensitivity in

heparin, as described above (data not shown). All subsequent RT-QuIC analyses included heparin-preserved whole blood that had undergone four freeze-thaw cycles.

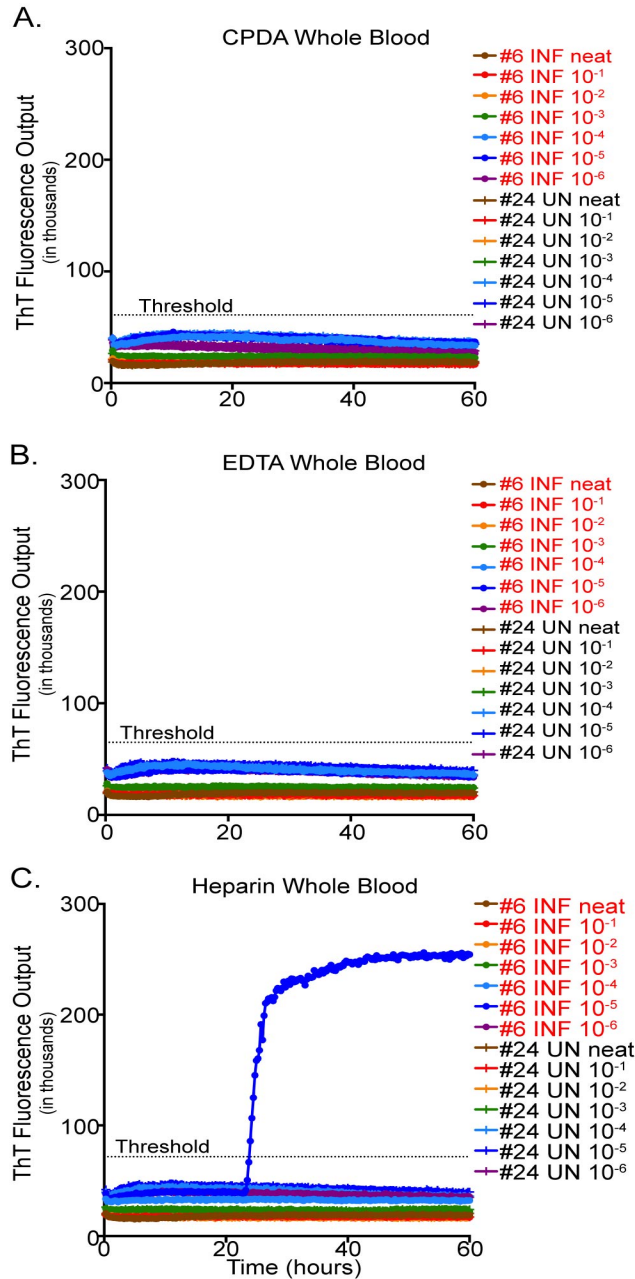


Figure 1.1 RT-QuIC analysis of whole blood collected in various anticoagulants.

Blood was collected from a CWD-infected and CWD-naïve white-tailed deer and preserved in one of three anticoagulants: CPDA, EDTA, or heparin. Serial blood sample dilutions (neat to 10^{-6}) were assayed by RT-QuIC for 60 hours and ThT fluorescence level above threshold determined positivity. Detection of PrP^C-converting activity for each replicate is shown for blood collected in CPDA (A), EDTA (B), and heparin (C).

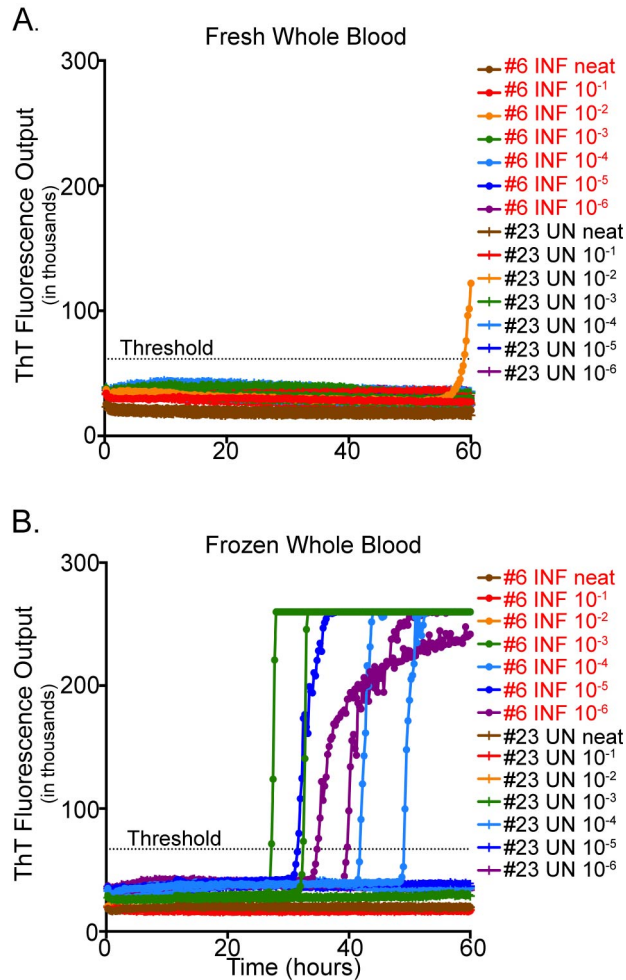


Figure 1.2 RT-QuIC analysis of fresh versus frozen whole blood.

Blood was collected from a CWD-infected and CWD-naïve white-tailed deer and aliquots were analyzed immediately (fresh) or frozen (-80C). Serial blood sample dilutions (neat to 10^{-6}) were assayed by RT-QuIC in duplicate for 60 hours and ThT fluorescence level above threshold determined positivity. Detection of PrP^C-converting activity for each replicate is shown for blood analyzed fresh (A) and frozen (B).

Effects of sodium phosphotungstic acid precipitation (NaPTA) on RT-QuIC PrP^D detection:

While blood that had been freeze-thawed revealed more PrP^C-converting activity than fresh blood, the results demonstrated an inconsistency in regards to the time required for a sample to become positive and dilutions that were positive. In addition to these inconsistencies, false-positive results were also witnessed. NaPTA precipitation was applied to heparin preserved whole blood that had undergone freeze-thaw cell lysis in an attempt to increase consistency of

positive samples, as well as the sensitivity and specificity of the RT-QuIC assay. With the improved sensitivity and specificity provided by NaPTA pretreatment, we were able to demonstrate reliable RT-QuIC results at a 10^{-2} dilution of CWD-infected whole blood, while NaPTA treated whole blood from a naïve individual remained conversion free (Figure 3). Samples were serially diluted, with the dilutional series for each animal being run in triplicate for 60 hours.

All of the remaining RT-QuIC analyses of TSE prion converting activity in historical and contemporary samples were conducted with heparin-preserved and freeze-thawed NaPTA-treated whole blood.

RT-QuIC comparison of CWD-positive brain versus NaPTA concentrated whole blood:

To evaluate the levels of PrP^D present in NaPTA concentrated whole blood samples, PrP^C-converting activity was compared to that detected in serial dilutions of CWD-positive white-tailed deer brain (Figure 4). NaPTA treated whole blood (500 µl starting volume of whole blood concentrated to 50 µl) diluted to 10^{-2} demonstrated PrP^D levels approximately equivalent to that measured in 10^{-6} - 10^{-7} dilution of CWD-positive brain. Equivalence was determined by comparison of the time to positivity for whole blood and brain samples.

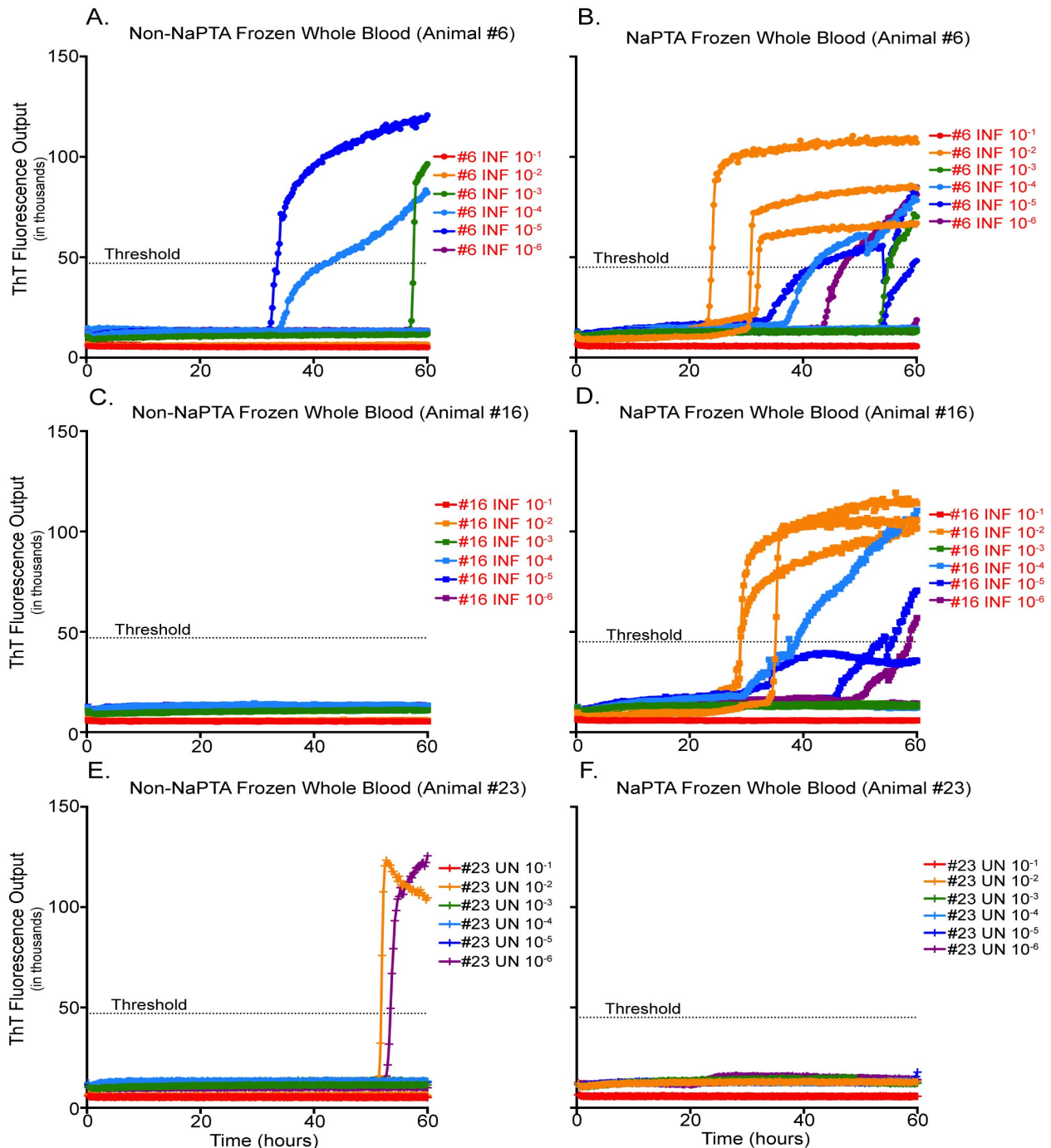


Figure 1.3 RT-QuIC analysis of samples before and after treatment with NAPTA. Samples were untreated or concentrated using NAPTA, serially diluted (neat to 10^{-6}), and assayed by RT-QuIC in triplicate for 60 hours. ThT fluorescence level above threshold determined positivity, each replicate is present. (A and C) Limited detection is seen in untreated blood samples. (B and D) Improved detection of PrP^C-converting activity is seen in blood samples precipitated with NAPTA from CWD-infected white-tailed deer. (E) Note increased false-positives in untreated samples from a CWD-naïve white-tailed deer. (F) No PrP^C-converting activity was seen in samples precipitated with NAPTA from a CWD-naïve white-tailed deer.

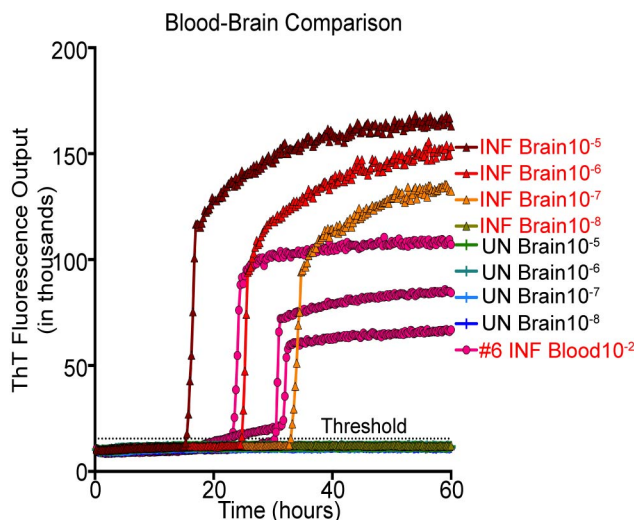


Figure 1.4 RT-QuIC comparison of brain and blood samples.

Ten percent (10%) brain homogenates were serially diluted (10^{-5} to 10^{-8}) and assayed by RT-QuIC for 60 hours. Blood samples were diluted to 10^{-2} and run in triplicate for 60 hours with ThT fluorescence level above threshold determining positivity. CWD-infected blood diluted 10^{-2} is seen to have similar levels of PrP^C converting activity as CWD-positive brain diluted 10^{-6} and 10^{-7} . UN= Uninfected; INF= Infected.

Detection of PrP^C-converting activity in CWD-infected cervid whole blood:

Twenty-two of 22 clinical and preclinical CWD-infected cervids (16 white-tailed deer and 6 muntjac deer) and 0/11 naive cervids (5 white-tailed deer and 6 muntjac deer) exhibited RT-QuIC PrP^C-converting activity in 7/8 or 8/8 replicates within 60 hours (Figure 5, Table 1). Samples were run two separate times to determine consistency of the RT-QuIC assay. Sample replicates were averaged on each plate and a positive threshold was set at five times the standard deviation of the negative control average.

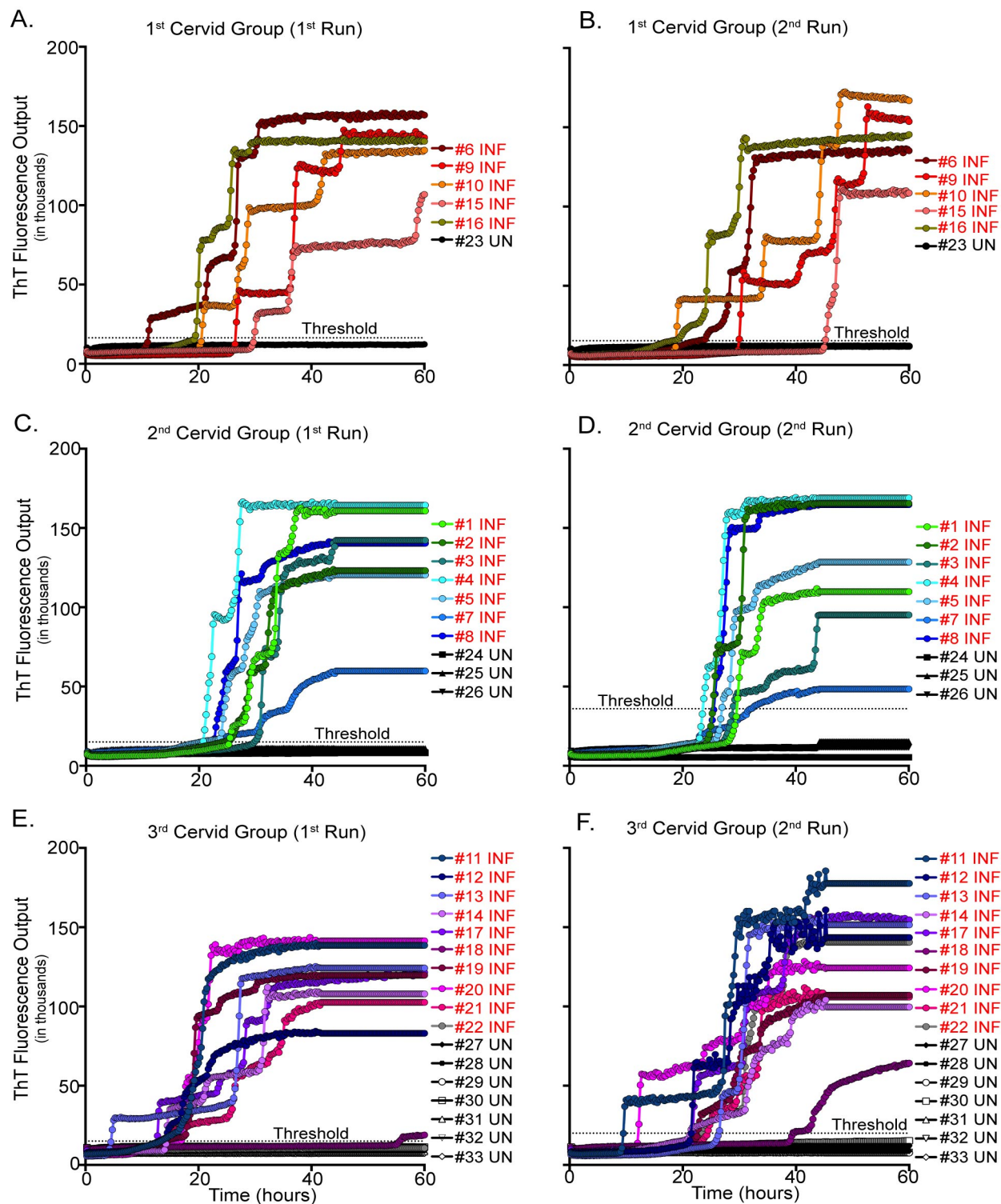


Figure 1.5 RT-QuIC analysis of cervid whole blood samples.

Blood samples were diluted to 10^{-2} and 8 replicates were analyzed over 2 runs of 60 hours, and positivity was determined by ThT fluorescence level above threshold. PrP^C-converting activity is demonstrated in 22 CWD-infected cervid blood samples, and is absent in all CWD-naïve samples (A-F). Each line is the average of four replicates for a specific animal. UN= Uninfected; INF= Infected.

Detection of PrP^C-converting activity in TME-infected hamster whole blood:

The hyper strain of transmissible mink encephalopathy (HY TME) was chosen for the RT-QuIC assay to determine the assays ability for PrP^D detection in various species and strains of TSEs. All HY TME-infected hamsters (n=21), ranging from 8 to 20 weeks post infection, exhibited RT-QuIC PrP^C-converting activity in 5/8 – 8/8 replicates within 60 hours, while all (n=7) of the age matched controls failed to seed RT-QuIC (Figure 6, Table 2). As above, each sample was run two separate times to determine consistency of the RT-QuIC assay. Sample replicates were averaged on each plate and a positive threshold was set at five times the standard deviation of the negative control average.

Immunohistochemistry confirmation of RT-QuIC results:

Immunohistochemistry was applied as a confirmation for the presence of PrP^D deposition in the brains of animals where PrP^C-converting activity was detected in blood. IHC was performed on both cervid and hamster TME-inoculated and mock-inoculated brains for detection of the disease associated isoform of the prion protein, PrP^D. PrP^D deposition was observed in TSE-infected animals, but not in mock-inoculated animals (Figure 7; Tables 1, 2).

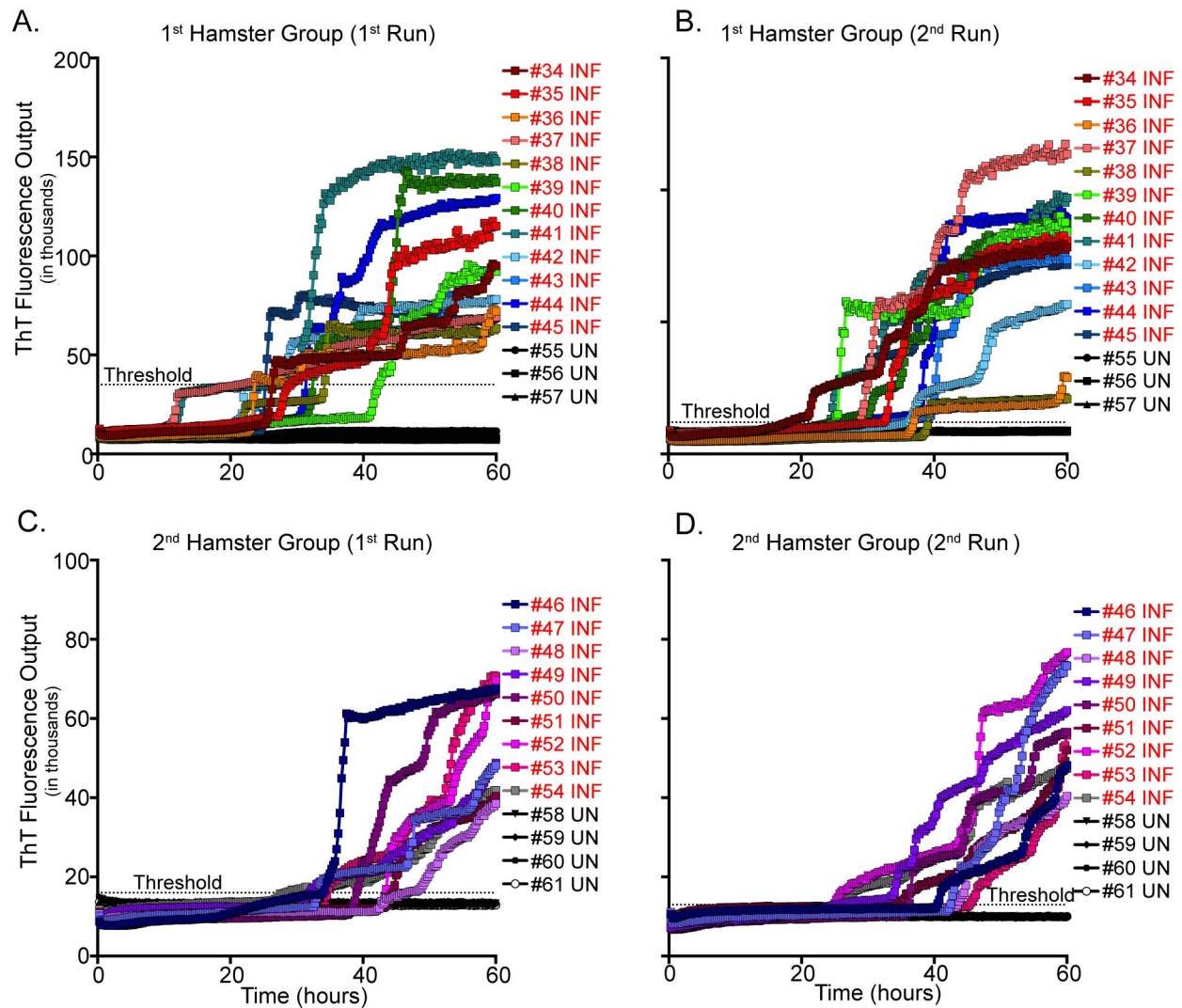


Figure 1.6 RT-QuIC analysis of hamster whole blood samples.

Blood samples were diluted to 10^{-2} and 8 replicates were analyzed over 2 experiments of 60 hours, and positivity was determined by ThT fluorescence level above threshold. PrP^C-converting activity is demonstrated in 21 TME-infected blood samples, and is absent in all TME-naïve samples (A-D). Each line is the average of four replicates for a specific animal. UN= Uninfected; INF= Infected.

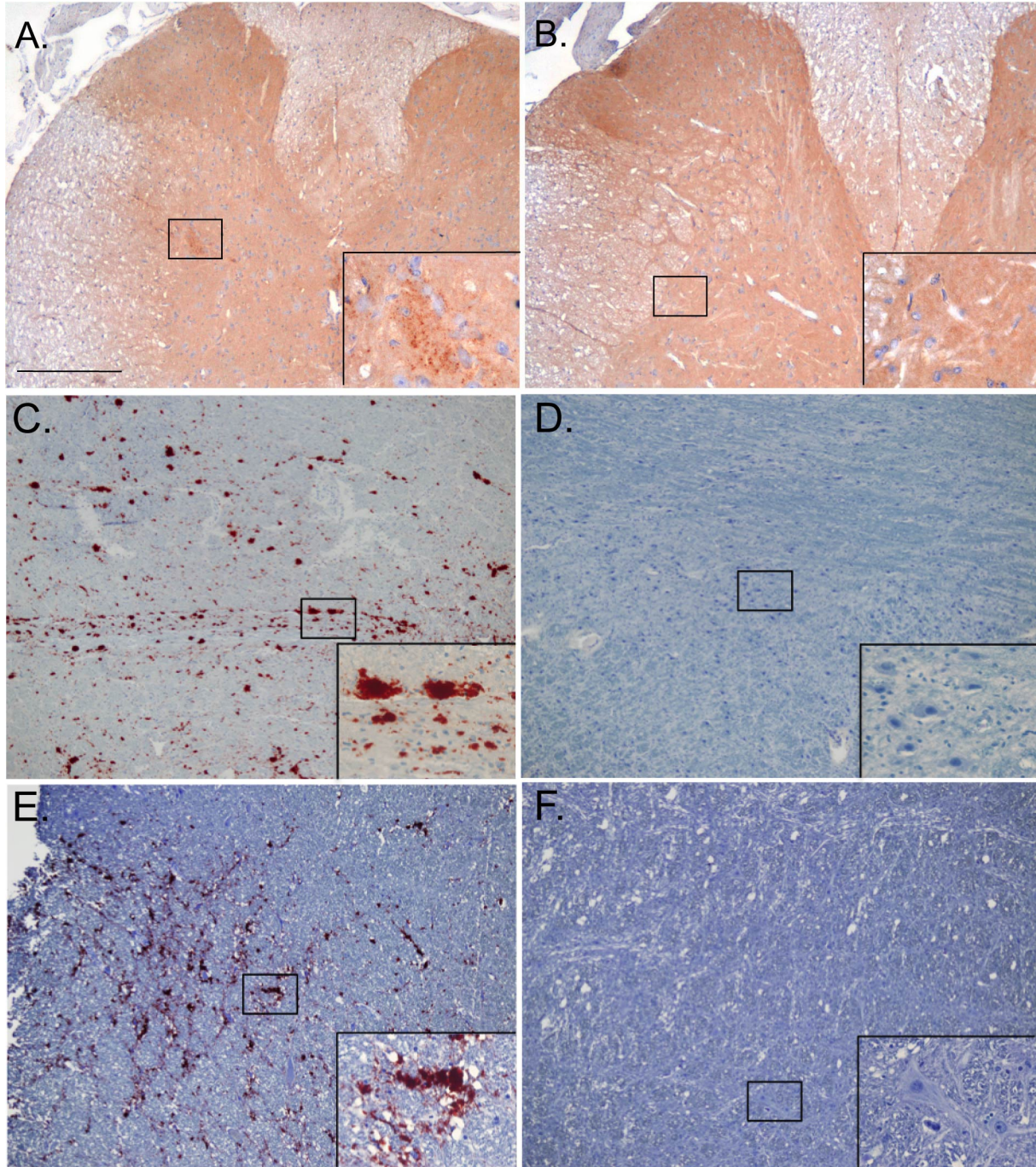


Figure 1.7 PrP^D detection in hamster, white-tailed deer and muntjac by IHC.

PrP^D immunoreactivity in a spinal cord tissue section from a hamster 16 weeks after extranasal inoculation with HY-TME (A) detected with antibody 3F4 and ABC solution. PrP^D immunoreactivity in the brainstem of CWD-infected white-tailed deer (C) and muntjac (E) detected with antibody BAR224 and AEC (3-amino-9-ethylcarbazole) substrate. No immunoreactivity was seen in the corresponding tissues of mock-inoculated controls (B, D and F). The boxed areas are enlarged 10x in the insets. Scale bar = 200 μ m.

Mouse and hamster bioassay sensitivity vs. RT-QuIC sensitivity:

To determine the brain equivalent sensitivity of RT-QuIC for TME and CWD samples, RT-QuIC analysis of serial dilutions of TSE-positive brain homogenates were compared to lethal dose bioassay titrations in HY TME-infected hamsters and CWD-infected mice.

Using bioassay in cervidized transgenic mice and the Reed-Muench method, the LD₅₀ titer for 1 ml of 10% CWD-positive brain homogenate was determined to be 10^{4.664}, or 4.62x10⁴ units/ml (calculated from values in Table 3). End point dilution analysis revealed a failure to cause disease in dilutions greater than 10⁻⁵. Serial dilutions of 10% homogenate CWD-positive brain homogenates in RT-QuIC demonstrated consistent positivity to a dilution of 10⁻⁶, with 50% converting activity detected in the 10⁻⁷ dilution (Figure 8A). SD₅₀ titer for the RT-QuIC assay was calculated for 1 ml of CWD-positive brain and was determined to be 10^{9.544}, or 3.5x10⁹ units/ml. These results indicate that the sensitivity of RT-QuIC for CWD detection is greater than animal bioassay.

The LD₅₀ for hamsters intracranially inoculated with HY TME was determined to be 10^{9.3}, as demonstrated previously by Kincaid, et al. [40]. Endpoint dilution analysis resulted in failure to cause disease in dilutions greater than 10⁻⁹. RT-QuIC analysis of the same HY TME brain homogenates revealed PrP^C-converting activity to 10⁻¹⁰ (Figure 8B). SD₅₀ titer for the RT-QuIC assay was calculated for 1 ml of HY TME-positive brain and was determined to be 10^{13.033}, or 1.08x10¹³ units/ml. This indicates that the sensitivity of RT-QuIC for HY TME detection is greater than animal bioassay.

Table 1.3 Bioassay of CWD-positive cervid brain in TgCerPrP mice

Dose (% brain homogenate)	# Clinical/total n	Days post inoculation (DPI) to clinical disease
10	7/9 ^A	137 ± 63 DPI
1	9/9	200 ± 29 DPI
0.1	8/9 ^B	220 ± 70 DPI
0.01	8/9 ^B	250 ± 68 DPI
0.001	7/9	397 ± 152 DPI
0.0001	1/9	335 DPI
0.00001	0/9	NA

DPI = Days post inoculation; ^A = 2/9 mice died for reasons unrelated to CWD infection; ^B = 1/9 mice died for reasons unrelated to CWD infection; NA = Not applicable

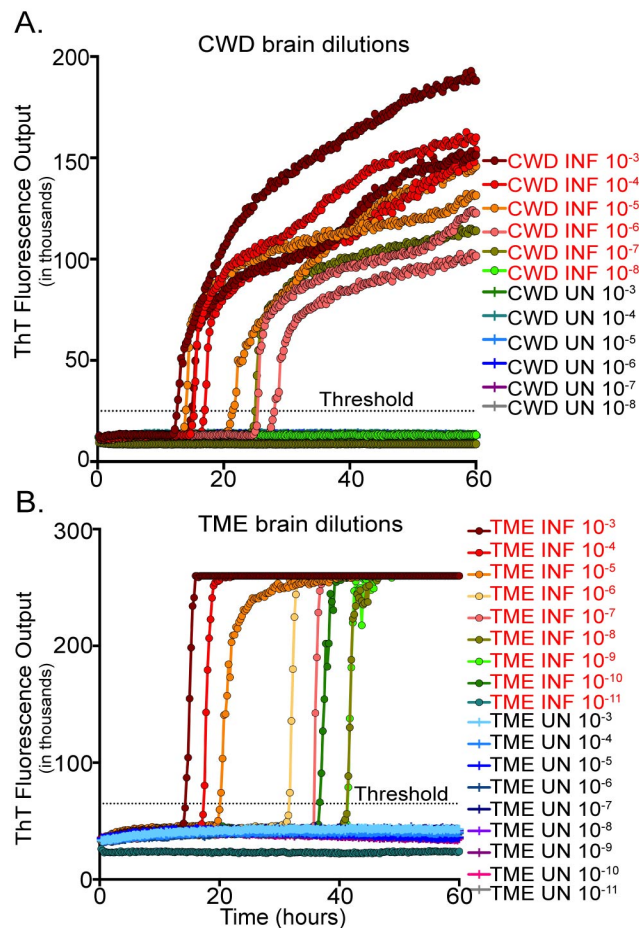


Figure 1.8 RT-QuIC analysis of serially diluted cervid and hamster brain samples. Brain samples were serially diluted 10⁻³ to 10⁻⁶ or 10⁻³ to 10⁻¹⁰ for cervids (A) and hamsters (B), respectively, and analyzed in RT-QuIC for 60 hours. A ThT fluorescence level above threshold determined positivity. Both cervid and hamster brains from positively inoculated animals demonstrated positivity in all dilutions, while all brain dilutions from naïve animals remained negative.

DISCUSSION

RT-QuIC analysis of whole blood collected in various anticoagulants:

Precedence for hematogenous spread of prions via transfusion has been well established with various TSEs, including: scrapie [11], CWD [13; 41], BSE in sheep [10] and vCJD [14; 16-19]. To date, few *in vitro* assays are capable of detecting prions present in the blood of infected individuals, and those that do can suffer from decreased sensitivity, possibly due to the presence of assay inhibitors [26; 42-44].

To assess whether anticoagulants affect PrP^D detection, we analyzed whole blood collected in CPDA, EDTA, and heparin. It has been demonstrated in previous work [45] that anticoagulant storage can affect the presentation of cellular PrP. Here, we have shown that whole blood collected in heparin, but not in CPDA or EDTA, elicited efficient *in vitro* RT-QuIC prion conversion. In addition to the conversion observed in heparin-preserved whole blood, it should be noted that only more dilute samples (10⁻⁵ dilution in particular) demonstrated PrP^C-converting activity. We suspect that this is due to the presence of inhibitory products in whole blood and that further diluting samples decreases the inhibition of these products.

It has been shown that polyanions enhance the amplification of prions in *in vitro* conversion assays, suggesting that they may contribute to conversion efficiency [46; 47]. Heparin, a polyanion, has previously been shown to enhance *in vitro* detection of PrP^D [48] and is thought to serve as a potential cofactor in prion propagation *in vivo* by acting as a scaffolding molecule or catalyst due to its highly negatively charged-glycosaminoglycan nature [49]. EDTA and CPDA owe their anticoagulant property to their ability to chelate calcium in blood. Chelating agents are widely used for scavenging metal ions [50] which may contribute to the

absence of PrP^C-conversion observed in blood collected in the two anticoagulants. However, it should be noted that detection of prions in CPDA blood has been observed following immunoaffinity capture and substrate replacement [29]. Further research is needed to determine the role anticoagulants play in inhibiting/facilitating RT-QuIC. We have demonstrated that preserving whole blood samples in heparin may facilitate *in vitro* prion detection without increasing false positives from uninfected samples.

RT-QuIC analysis of fresh versus frozen whole blood:

To assess the feasibility of using historical frozen samples for future analysis of blood-borne prions, we evaluated the effects of freezing blood prior to RT-QuIC. We have demonstrated that the freeze-thaw cycle enhances RT-QuIC blood-borne prion detection sensitivity, facilitating *in vitro* prion detection at earlier time points with a more robust amplification than samples that did not undergo the freeze-thaw process. There is compelling evidence for the accumulation of aggregated misfolded prion isoforms in the cytoplasm of infected cells [51; 52] and it is hypothesized that these aggregates are released from the cell as lysis occurs. Thermal shock on whole blood samples damages the cell membrane and initiates hemolysis [53; 54], which is thought to release intracellular components. Cell lysis of blood collected from TSE-infected animals, associated with repeated freeze-thaw cycles, may liberate sufficient prions to enhance *in vitro* nucleation and thus the detection of PrP^C-converting activity.

Effects of sodium phosphotungstic acid precipitation on RT-QuIC PrP^D detection:

It has been suggested that there are components present in bodily fluids that interfere with or inhibit prion conversion and thus *in vitro* detection of the aberrant form of the prion protein

[55; 56]. Various groups have attempted to solve this problem using different concentration methods. Using immunoprecipitation coupled with RT-QuIC, Orrú et al. [29] were able to establish *in vitro* detection of PrP^C-converting activity in plasma and serum samples from scrapie-infected hamsters. Morales et al. [57] demonstrated that the use of varying concentrations of sarkosyl could concentrate PrP^D present in tissue and fluid samples. Wadsworth and colleagues [35; 58] have shown that sarkosyl, coupled with the use of sodium phosphotungstic acid, enhances the isolation of both PrP^C and PrP^D from bodily fluids. Some groups have reported an inhibitory effect on amyloid formation when using NaPTA precipitation [59]; however, this was not our experience (Figure 3).

Using NaPTA precipitation we were able to concentrate hematogenous prions to a more detectable level and/or remove assay inhibitors, augmenting our ability to directly detect prions in whole blood. Samples not receiving NaPTA treatment took longer to convert PrP^C, and did so only in more dilute samples (Figure 3A). Samples that received treatment with NaPTA precipitation revealed PrP^C-converting activity earlier, and exhibited positivity in more concentrated samples (Figure 3B). We conclude that NaPTA precipitation may remove potential assay inhibitors that are present in blood, allowing detection of converting activity at more concentrated dilutions thus decreasing false negatives (Figure 3C, D). In addition to these observations, we attempted sonication of the NaPTA product prior to serial dilution to determine if this aided in the observation of a dose-response. While this method slightly increased the number of later dilutions expressing PrP^C-converting activity and aided in the consistency of when they crossed the positivity threshold (data not shown), the effect witnessed was not great enough to alter our decision to use the 10⁻² dilution for all subsequent experiments.

With the application of an anticoagulant that facilitates prion conversion *in vitro*, the freeze-thaw cell lysis and NaPTA precipitation, we have optimized the RT-QuIC assay for efficient detection of PrP^D in whole blood samples, thus we are calling our new protocol whole blood optimized (WBO) RT-QuIC. NaPTA precipitation increased consistency, the number of positive replicates and decreased the assay time required to initiate PrP^C conversion/detection in whole blood harvested from TSE-infected animals while limiting false positive PrP^C-converting activity in samples from uninfected animals (Figure 3).

RT-QuIC comparison of CWD-positive brain and NaPTA concentrated whole blood:

Many groups have developed quantitative *in vitro* methods to analyze the levels of PrP^D present in various tissues and bodily fluid samples. Murayama et al. [60] used PMCA to establish a direct comparison of PrP^D levels in buffy coat and plasma to PrP^D levels seen in serial dilutions of TSE-infected brain by analyzing which round of PMCA samples began demonstrating positivity. Other laboratories [26; 56; 61] have reported quantitative and semi-quantitative methods of PMCA to determine the levels of PrP^D in blood and urine by comparing to the amount of amplifiable PrP^D present in TSE-infected brain. Castilla et al. [26] were able to demonstrate that PMCA amplifiable prions in buffy coat collected from 1 ml of scrapie-adapted hamster blood contained roughly 0.1-1 pg of PrP^D molecules. Our RT-QuIC results indicate that 2 µl of a 10⁻² dilution (0.5 ml of whole blood NaPTA concentrated 10-fold, further diluted to 10⁻²) contained PrP^D levels equivalent to those seen in 0.02 ng - 0.2 ng of CWD-positive brain (Figure 4).

Detection of PrP^C-converting activity in CWD-infected cervid whole blood:

Wilham et al. [30] demonstrated that the RT-QuIC assay has the ability to detect prions in tissue samples with similar sensitivity as bioassay (~ 1 lethal dose), rendering it appropriate for the detection of PrP^D in bodily fluids such as blood and saliva. RT-QuIC assay efficacy for CWD-infected whole blood was evaluated following pretreatment to augment the release of prions from carrier cells and minimize inhibitory factors (freeze-thaw/NaPTA). We have demonstrated that our optimized RT-QuIC assay is sufficiently sensitive to detect PrP^C-converting activity in whole blood harvested from preclinical and clinical IHC/Western blot-confirmed CWD-infected animals. Furthermore, our optimized RT-QuIC assay has demonstrated the ability to detect PrP^C-converting activity in CWD-inoculated animals prior to the mid point between inoculation and clinical disease.

Using PMCA for the detection of PrP^D in the blood of scrapie-infected hamsters, Saa et al. [25] reported sensitivity levels of 80% for clinical animals, and up to 60% for preclinical animals. Orrú et al. demonstrated even greater sensitivity for PrP^D in blood plasma of scrapie-infected hamsters using immunoprecipitation coupled with RT-QuIC [29]. Utilizing our optimized RT-QuIC assay for cervid whole blood, we have shown that our assay exhibited sensitivity levels of 93.8% for clinical animals and 92.2% for preclinical animals while maintaining 100% specificity. These results reveal the potential of RT-QuIC as a reliable *in vitro* assay for blood-borne prion detection.

Detection of PrP^C-converting activity in TME-infected hamster whole blood:

Utilization of hamster models for the propagation and detection of hematogenous PrP^D have been used extensively [25; 26; 29; 51; 60; 62], primarily with scrapie infections. Previous

to this study, RT-QuIC had not been used to probe for PrP^C-converting activity in whole blood of TME-infected hamsters. To ensure that the detection of RT-QuIC blood-borne PrP^D detection was not exclusive to CWD, we analyzed whole blood harvested from IHC-confirmed TME-infected and mock-infected hamsters. We have demonstrated PrP^C-converting activity in preclinical TME-infected hamsters with 94.4% sensitivity and 100% specificity. We have also shown that the WBO RT-QuIC assay possesses the ability to detect PrP^D in the blood of TME-infected hamsters prior to the midpoint between inoculation and clinical disease.

These observations reveal that RT-QuIC is consistently more sensitive in detection of hematogenous PrP^D in preclinical animals than previously reported for PMCA [25]. Thus, the WBO RT-QuIC assay may be applicable for the detection of prionemia in multiple species (animals/humans).

Implications for in vitro detection of blood-borne prion disease:

The development of a reliable *in vitro* blood-borne TSE-detection assay would have significant advantages for both human and animal populations and may provide a stepping-stone for the development of diagnostic assays for other protein misfolding diseases. To date, various *in vitro* assays have been developed with the goal of detecting prions present in blood [63]. Of particular note are sPMCA [25; 26], a ligand based assay developed to detect hematogenous prions [42], and immunoprecipitation enhanced RT-QuIC [29]. However, demonstrating satisfactory sensitivity and specificity with these assays has been a challenge.


We have demonstrated *in vitro* detection of prionemia in CWD and TME-infected hosts during both pre-clinical and clinical phases of disease, establishing the merits of RT-QuIC as an effective antemortem diagnostic tool. Early detection and screening applications will provide a

means to detect asymptomatic carriers of TSE disease in the human donor blood and tissue-pools, thus indicating which samples should be eliminated. The ability to detect infected blood will aid in establishing monitoring parameters for TSE intervention/therapeutic strategies and provide domestic and wildlife herd management professionals with a live test for TSE surveillance.

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CHAPTER 2

Temporal parameters of prionemia in hamsters and deer following oral, nasal, or blood inoculations: from minutes to terminal disease

OVERVIEW

Infectious prions have been shown to traverse epithelial barriers to gain access to the circulatory system, yet the details of prion entry and persistence in the blood remains unknown. Conversion-competent blood-borne prions have been demonstrated in deer and hamsters infected with a transmissible spongiform encephalopathy (TSE) using whole blood real time-quaking induced conversion (wbRT-QuIC). Here we employ wbRT-QuIC to analyze whole blood collected from mucosal or intravenous TSE-inoculated deer and hamsters, beginning within minutes of inoculation and extending to the onset of clinical symptoms. Our results demonstrate the presence of conversion-competent prions in the blood of all TSE-inoculated hosts as early as 15 minutes post inoculation (pi) following peripheral inoculation. The presence of conversion-competent prions changed between 24-72 hrs pi with conversion-competent prions being detected in 22.92% - 6.25% replicates, respectively, likely representing the point source inoculum. While an eclipse phase was not identified, the clearance of prions was followed by a subsequent increase in detectable levels of hematogenous conversion-competent prions as disease progressed. These results indicate the occurrence of a primary and a secondary prionemia following different routes of exposure. This is the first report of the detection of blood-borne

prions throughout the complete incubation period of TSE disease, yielding evidence for the establishment of an asymptomatic carrier state within minutes of TSE exposure.

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are infectious and inevitably fatal neurodegenerative diseases that affect animals, including humans. TSEs are characterized by the conversion of the normal cellular prion protein (PrP^C) into the aberrant misfolded disease associated conformer (PrP^{Sc}) and are transmitted by various routes of exposure to PrP^{Sc} present in tissues[1-8] or bodily fluids[9-20]. Prion diseases are also transmitted by contaminated soils/fomites[21-23], inadvertent medical exposure[24], and by blood transfusion[25-27]. A long asymptomatic phase where infectious prions are present in tissues and fluids of infected hosts has been identified for several TSEs including: chronic wasting disease (CWD) in cervids[17; 18], scrapie in sheep[28], bovine spongiform encephalopathy (BSE) in cattle[29], transmissible mink encephalopathy (TME) in hamsters[19; 30], and Kuru and variant Creutzfeldt-Jakob disease (vCJD) in humans[31; 32].

Prion trafficking within the central nervous system (CNS) and peripheral organs has been explored[33-40]. Due to limitations in assay sensitivity and lack of longitudinal blood sample archives spanning the entirety of TSE disease, the role hematogenous prions play in prion pathogenesis is poorly understood. Prions cross the intestinal epithelium following oral inoculation and infect the lymphoreticular system (LRS), the earliest site of PrP^{Sc} deposition and replication[40-46], yet it is unknown how PrP^{Sc} infiltrates autonomic nerves. Blood transmission dynamics and disease progression are remarkably similar for many prion diseases; however, the

fate of prions in the circulatory system immediately after initial exposure and during the asymptomatic disease course is unknown.

Infectious hematogenous prions have been demonstrated in CWD-infected cervids[22] and hamsters infected with the hyper (HY) strain of hamster-adapted TME[47], which is similar to what is described for vCJD-infected humans. CWD, the only TSE found in native wildlife populations, is efficiently transmitted among free-ranging and captive cervid populations, and is found in 23 U.S. states, 2 Canadian provinces, and in South Korea[48]. While it is unknown how CWD has spread throughout North America, numerous studies have characterized intra-host CWD trafficking with strong emphasis on the LRS, as well as potential shedding through urine, feces, and saliva[22; 41; 49; 50], all of which may gain infectivity from the blood. Studies conducted with TSE-infected hamsters have provided valuable insights into prion infiltration across mucosal surfaces, trafficking to the LRS, and subsequent neuroinvasion[30; 51-54]. These LRS-associated prion diseases (CWD and HY-TME) are helpful for studies exploring the biological significance of hematogenous prions.

To better understand the pathogenesis of prion diseases, we determined the temporal distribution of prions in blood collected from TSE-infected animals throughout the entirety of disease (0-100% of the disease state). By analyzing whole blood for the presence of PrP^C-converting activity collected minutes post inoculation to terminal disease, we sought to gain insight into how prions are trafficked throughout the body, when and how they are shed, and the role that blood-borne prions play in neuroinvasion.

MATERIALS AND METHODS

Ethics statement:

Guidelines for animal care and use, issued by the United States Department of Agriculture (USDA), National Institutes of Health (NIH), and the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), were adhered to for all animal work. Colorado State University (CSU) Institutional Animal Care and Use Committee (IACUC) (approval numbers 02-151A, 08-175A, 10-2189A, 11-2615A, 11-2622A, 13-4444A, and 14-4890A) approved all animal protocols. All hamster procedures were approved by the Creighton University IACUC and were in compliance with the NIH *Guide for the Care and Use of Laboratory Animals*.

White-tailed deer, muntjac deer and Syrian hamster source:

All animals were housed in BSL2+ indoor facilities where strict husbandry and quality assurance were maintained to assure assessment of point source inoculums. Protective clothing, sample instruments, and waste disposal were specific to each animal room to exclude any possibility of cross contamination caused by fomites or contact.

White-tailed deer: White-tailed deer (*Odocoileus virginianus*) were provided through collaboration with the Warnell School of Forestry and Natural Resources at the University of Georgia (Athens, GA) where CWD has not been detected. All animals were adapted to humans and indoor facilities prior to transport to CSU. Upon arrival, deer were immediately isolated in indoor research facilities never contacting the Colorado environment.

Reeves' muntjac deer: Reeves' muntjac deer (*Muntiacus reevesi*) (also called muntjac deer) were sourced from Cervid Solutions Inc. (Tellico, TN) in a region where CWD has not been detected. All animals were transported to CSU and placed into an isolated indoor facility without contacting Colorado soil.

Syrian hamsters: Male 10-11 week old Syrian hamsters (*Mesocricetus auratus*) were obtained from Harlan Sprague Dawley (Indianapolis, IN) and group-housed in separate cages. All animals were housed in sterile TSE-free cages before inoculation.

Cervid inoculations and tissue collections:

To maximize animal use, samples were collected from previous and contemporary studies were analyzed to study the full course of disease, from point of TSE-exposure through terminal TSE disease.

White-tailed deer inoculations: Cervids were anesthetized prior to CWD inoculation and sample collection as previously described[55]. CWD-naïve, white-tailed deer (total n=34) received the following CWD-positive inocula (Table 1): 1) n=6, 2 ml of a 5% (w/v) brain homogenate aerosol-administered[6]; 2) n=19, 1.0 g of a 10% (w/v) brain homogenate orally (PO); 3) n=4, 0.55 g of a 10% (w/v) brain homogenate intravenously (IV); 4) n=1, 225 ml of CWD-positive whole blood IV[9]; or 5) n=4, 0.55g of a 10% (w/v) brain homogenate PO (0.5 g) and intranasal (IN, 0.05 g). Negative control white-tailed deer (total n=6) were inoculated with sham material (CWD-negative brain homogenate) by the same routes described above.

Muntjac deer inoculations: Prior to inoculation and sample collection, muntjac deer were anesthetized as previously described[20; 55]. CWD-naïve muntjac deer (total n=10) were

inoculated with CWD-positive material via the following routes: 1) n=6, 1.0 g of a 10% (w/v) brain homogenate administered PO/SQ[20]; 2) n=2, 20 ml fresh whole blood (collected in citrate phosphate dextrose-adenine (CPDA)) administered IV; or 3) n=2, 0.55 g of a 10% (w/v) brain homogenate PO (0.5 g) and IN (0.05 g). Negative control muntjac deer (total n=8) received sham material (CWD-negative brain homogenate) via PO/IN inoculation (n=2), PO inoculation (n=2), or were uninoculated (n=4).

Cervid blood and tissue collections: All data for this report were generated from new 1 ml aliquots of whole blood. Ten (10) ml of whole heparinized (1 ml; 200 units heparin/ml) and CPDA (1 ml; 14% CPDA blood tubes) blood was collected from each cervid at various time points: 15, 30, and 60 minutes post inoculation (pi); 24, 48, and 72 hours pi (0.001%-0.3% of disease course); and 1-34 months pi and at terminal clinical disease (~4%-100% of disease course). Whole blood samples—taken at time points from above—were collected from negative control cervids. All samples were preserved in heparin or CPDA were frozen and stored in 1 ml aliquots at -80°C.

At study termination all cervids were humanely euthanized in accordance to CSU IACUC protocols. The obex at the medulla oblongata and multiple additional lymphoid and non-lymphoid tissues were collected from each cervid. Each tissue was divided in half to allow one half to be frozen at -80°C and the other to be fixed in 10% neutral buffered formalin or paraformaldehyde-lysine-periodate for later processing.

Table 2.1 Cervid and hamster inoculation data and TSE disease progression

Animal Type	Sample Size	Inoculation Route	Inoculum	Inoculum Amount	Onset of Symptomatic Disease
Reeves' muntjac deer (Muntjac deer)	n=18	IV	CWD+ blood n=2	20 ml CWD+ whole blood	N/A
		PO/SQ	CWD+ brain homogenate n=6	1.0 g of a 10% homogenate	20-25 mo-pi
			Sham material n=2		N/A
		PO/IN	CWD brain homogenate n=2	0.55 g of a 10% homogenate	N/A
			Sham material n=2		N/A
		Uninoculated	N/A n=4	N/A	N/A
White-tailed deer	n=39	IV	CWD+ brain homogenate n=4	0.55 g of a 10% CWD+ brain homogenate	N/A
		PO	CWD+ brain homogenate n=19	1.0 g of a 10% homogenate	13-23 mo-pi
			Sham material n=2		N/A
		PO/IN	CWD+ brain homogenate n=4	.55 g of 10% CWD+ brain homogenate	N/A
		IV/PO/IN	Sham material n=1	1.1 g of a 10% sham homogenate	N/A
		Aerosol	CWD+ brain homogenate n=6	2 ml of a 5% homogenate	17-18 mo-pi
			Sham material n=3		N/A
Golden Syrian hamster	n=90	EN	HY-TME brain homogenate n=54	10 µl of a 10% homogenate—5 µl/nostril	20 wpi
			Sham material n=36		N/A

EN=Extranasal; PO=Oral; SQ=Subcutaneous; IN=Intranasal; IV=Intravenous; HY-TME=Hyper strain of transmissible mink encephalopathy; N/A=Not applicable; wpi=Weeks post inoculation; mo-pi=Months post inoculation

Hamster inoculations and tissue collections:

Hamster inoculations: As hamsters are obligate nose breathers, Syrian hamsters were anesthetized and inoculated extranasally (EN) as previously described[30]. Hamsters (n =54) were inoculated with either 10 µl HY-TME—5 µl to each nostril—with a 10% (w/v) brain homogenate containing an LD₅₀ of 10^{6.8}, or sham inoculum (n=36) as previously described[51].

Hamster blood and tissue collections: Blood was collected from hamsters—3 HY-TME-inoculated and 2 sham-inoculated—at selected time points: 15, 30, and 60 minutes pi; 24 and 72 hours pi; 5, 7, and 10 days pi (5%-10% of disease course); and at 2 week intervals from 2 to 20 weeks pi (14%-100% of disease course)(results for 8-20 week pi hamsters previously reported by Elder et al.[55]). After anesthetization, blood was collected via cardiac puncture and stored in heparin blood tubes (200 units/ml) for preservation at -80°C.

Cervid and hamster brain tissue homogenization:

Ten percent (10%) TSE-positive and negative brain tissue homogenates were prepared from the obex region of each brain. Brain tissue (0.05 g) in 0.5 ml homogenate buffer (1X PBS + 0.1% Triton-X 100 [Sigma-Aldrich]) were added to 1.5 ml tubes containing 0.5 mm diameter ZrO₂ beads and homogenized by Bullet BlenderTM (Next Advance) for 5 minutes at a setting of 10. Homogenates were stored in 20 µl aliquots at -80°C.

Recombinant protein preparation:

Recombinant protein was expressed and purified as previously described[49; 56]. In brief, a truncated recombinant Syrian hamster PrP (SHrPrP 90-231; from Byron Caughey at Rocky Mountain Laboratories in Hamilton, MT) was expressed via auto induction (EMD

Biosciences) in Rosetta strain *Escherichia coli*. The recombinant protein was isolated from inclusion bodies, purified and refolded over a Ni column. Final purified and dialyzed protein was adjusted to a final concentration between 0.3 and 0.7 mg/ml and refrigerated in 1 ml aliquots.

Whole-blood-optimized real-time quaking-induced conversion (wbRT-QuIC):

RT-QuIC was optimized for use with whole blood samples as previously described[55]. Aliquots of whole blood (1 ml) were subjected to four freeze/thaw cycles of freezing at -80°C for 30 minutes and thawing at 22°C for 60 minutes—prior to bead homogenization (described above). Following freeze/thaw cell lysis, a modified version[55] of the sodium phosphotungstic acid (NaPTA) precipitation protocol, described by Wadsworth et al.[57], was used to concentrate PrP^C and PrP^{res}. 500 µl of blood was treated with 4% sarkosyl (Sigma-Aldrich), benzonase (298 U/ml), and 4% (w/v) sodium phosphotungstate. Samples were centrifuged, supernatants removed, and the resulting pellets resuspended in 50 µl 0.1% sarkosyl (v/v) before being analyzed by wbRT-QuIC.

wbRT-QuIC[55] was performed to detect prions in processed blood samples. Positive assay controls consisted of serial dilutions of 10% brain homogenates (10^{-4} - 10^{-7}) and processed blood (10^{-2}) from TSE-infected animals. Negative assay controls consisted of identical serial dilutions of brain homogenates and processed blood from uninfected or mock-infected animals. In brief, RT-QuIC reactions (brain controls in triplicate and blood samples 8 replicates/sample) were set up in 96-well plate clear bottom optic plates (Nalgene Nunc) and placed in a BMG Fluostar fluorescence plate reader for 62.5 hours at 42°C (250 cycles). Cycles consisted of 1 minute of shaking and 1 minute of rest for 15 minutes with a ThT fluorescence measurement

taken at the end of each cycle. Data were processed using Microsoft Excel (Microsoft Inc.) prior to graph production with Prism 6 (GraphPad Prism).

Rate analysis of wbRT-QuIC data:

Data for all replicates of each sample were analyzed to determine the time when they became positive. Samples were deemed positive for PrP^C-converting activity if their fluorescence output crossed the set threshold (average of the negative control fluorescence plus 5 times the standard deviation). The rate of conversion to PrP^{Sc} was calculated for each replicate as: $Rate = \frac{1}{time\ to\ threshold}$. Replicates for each sample were averaged together and graphed using Prism 6.

Calculations:

The rate at which conversion-competent blood-borne prions were generated in RT-QuIC was compared to those generated from brain tissue harvested from terminal clinical PO- and aerosol-inoculated cervids (10^{-6} - 10^{-8})[58], or EN-inoculated hamsters (10^{-5} - 10^{-10}). Ten percent (10%) brain homogenates were serially diluted 10-fold and 2 μ l of each dilution was seeded into the RT-QuIC assay. Each dilution of brain homogenate was equated to a ng quantity as previously described[55]; e.g. 0.1 g brain tissue/ml diluted to 10^{-5} = $(10^{-9}\text{ g}/\mu\text{l})(2\ \mu\text{l}) = 2 \times 10^{-9}$ g brain tissue = 2 ng brain tissue equivalents. The rate at which conversion-competent prions were formed in brain tissue from CWD orally-inoculated cervids was generated by averaging 72 replicates (8 replicates per animal; 9 animals); CWD aerosol-inoculated animals (48 replicates; 8 replicates per animal; 6 animals); HY-TME extranasal-inoculated animals (32 replicates; 16 replicates per animal; 2 animals).

We calculated the amount of blood spiked into one wbRT-QuIC reaction (2 μ l of spike) to be the equivalent of 0.02 μ l of whole blood: 1) 500 μ l of whole blood was concentrated to 50 μ l using NaPTA; 2) 5 μ l of NaPTA product was serially diluted 1/100; 3) 2 μ l of the 10^{-2} dilution was seeded into 98 μ l of substrate. The rate of amyloid formation for blood was also compared to reaction rate for 1 LD₅₀ of brain homogenate, as previously described[58], to estimate the LD₅₀ of blood at that time point. The rate of amyloid formation for blood at a given time point was converted to the mass equivalent of brain using the following reaction rate equation:

$y = m \log(x) + b$. The LD₅₀ for 0.02 μ l of blood was then calculated as $\frac{\text{mass equivalent of brain}}{\text{mass of brain causing LD}_{50}}$.

RESULTS

To better understand the biological significance of prionemia in prion diseases, we analyzed blood collected from TSE-infected animals throughout the incubation period. The results for each time point are reported as the percentages of the total replicates displaying positivity from that route of exposure, or as the rate of PrP^{Sc} conversion in relation to CWD or HY-TME positive brain homogenate.

CWD prionemia:

Mucosal exposure: As identical results were observed in muntjac deer and white-tailed deer, results for the two species were combined. To examine trans-mucosal entry of CWD, we examined whole blood for hematogenous prions post oral or aerosol mucosal exposure (PO/SQ animals were pooled with PO animals due to the limited exposure via SQ). In PO-inoculated animals PrP^C-converting activity was observed in the blood at 15 min-pi (28.24% of replicates

from 17/17 cervids), with greater detectable converting activity (90.07%) observed at 30 min-pi–0.002% of the disease course (Table 2; Fig.1A). Between 0.09 -0.3% disease course (24-72 hrs), detectable converting activity changed from 22.92% of replicates (6/6 deer) to 6.25% of replicates (4/4 deer) (p-value<0.0001) (Table 2; Fig.1A). PrP^C-converting activity did not fall below assay detection levels during this time. Blood from all animals receiving mock inoculum remained free of PrP^C-converting activity (Fig.1A).

We further analyzed whole blood collected from cervids throughout the course of infection. In middle-stage infection samples we observed replication competent prions (12.5% of replicates) as early as 5% of the disease course (1 mo-pi) followed by a steady increase in the detection of nascent PrP^C-converting activity in blood harvested from CWD orally-exposed cervids through the onset of clinical disease (15-22 mo-pi; 77.78-84.38%) (Table 2; Fig.1B; 2A,C). PrP^C-converting activity was detected in blood harvested from aerosol-exposed cervids as early as 3 mo-pi and reached 100% of replicates of 100% of animals around 60% of the disease course (15 mo-pi) and was sustained throughout the study (deer terminated at 17.5-34 mo-pi) (Table 2; Fig.1B). The ability to detect hematogenous prions was similar in animals receiving inoculum by either oral or aerosol routes of administration.

Intravenous exposure: To ascertain prionemia post-transfusion, blood harvested following IV-inoculation was assessed for conversion competent prions by wbRT-QuIC. CWD-IV-inoculated animals displayed high levels of conversion competent hematogenous prions at 15 min-pi (100%), which remained present through 24 hpi. Subsequent to this initial detection, detection of PrP^C-converting activity between 0.09% and 0.3% of the disease course changed from 93.75% to 33.33% (Table 2; Fig.1A), mimicking the initial trajectory observed in mucosal-exposed cervids. Upon analysis of longitudinal samples collected from IV-inoculated animals,

nascent amplification-competent hematogenous prions were noted (12.5% of replicates), at 16% of the disease course (2 mo-pi), continuing through terminal disease (100% of replicates from 50-100% of the diseases course) (Table 2; Fig.1B).

Table 2.2 Complete cervid prionemia detection data

		Inoculation Route								
		IV			PO			Aerosol		
		Ave. positive replicates (8 per animal)	SD	<i>n</i>	Ave. positive replicates (8 per animal)	SD	<i>n</i>	Ave. positive replicates (8 per animal)	SD	<i>n</i>
Immediate Collections	0 min pi	0.0	0.0	6	0.0	0.0	17	ND	ND	ND
	15 min pi	8.0	0.0	5	2.26	1.09	17	ND	ND	ND
	30 min pi	7.8	0.4 5	5	7.21	1.66	17	ND	ND	ND
	60 min pi	7.75	0.5	4	4.72	0.84	9	ND	ND	ND
	24 hpi	7.5	0.7 1	2	1.83	1.5	6	ND	ND	ND
	48 hpi	5.25	0.9 6	4	1.17	0.5	6	ND	ND	ND
	72 hpi	2.66	1.1 5	3	0.5	0.71	4	ND	ND	ND
Longitudinal Collections	1 mo pi	ND	ND	ND	1.0	0.5	3	ND	ND	ND
	2 mo pi	1.0	0.0	1	ND	ND	ND	ND	ND	ND
	3 mo pi	2.0	0.0	1	1.18	0.61	11	2.83	0.98	6
	6 mo pi	8.0	0.0	1	2.09	0.83	11	5.67	1.03	6
	9 mo pi	ND	ND	ND	3.18	1.27	11	7.33	0.52	6
	10 mo pi	ND	ND	ND	5.43	1.51	7	ND	ND	ND

12 mo pi	8.0	0.0	1	6.09	1.14	11	7.66	0.52	6
15 mo pi	ND	ND	ND	6.22	1.5	9	7.33	0.82	6
16 mo pi	ND	ND	ND	7.5	0.71	2	7.83	0.41	6
17 mo pi	ND	ND	ND	ND	ND	ND	8.0	0.0	6
18 mo pi	ND	ND	ND	ND	ND	ND	7.83	0.41	6
19 mo pi	ND	ND	ND	ND	ND	ND	8.0	0.0	6
20 mo pi	ND	ND	ND	ND	ND	ND	8.0	0.0	6
21 mo pi	ND	ND	ND	ND	ND	ND	8.0	0.0	6
22 mo pi	ND	ND	ND	6.75	1.13	8	8.0	0.0	6
23 mo pi	ND	ND	ND	ND	ND	ND	8.0	0.0	6
25 mo pi	ND	ND	ND	6.6	0.55	5	8.0	0.0	6
28 mo pi	ND	ND	ND	7.6	0.5	5	ND	ND	ND
30 mo pi	ND	ND	ND	7.5	0.55	4	ND	ND	ND
31 mo pi	ND	ND	ND	7.5	0.71	2	ND	ND	ND
34 mo pi	ND	ND	ND	8.0	0.0	1	ND	ND	ND

SD=Standard Deviation; *n*=Number of animals sampled; IV=Intravenous inoculation; PO=Oral inoculation; min-pi=Minutes post inoculation; hpi=Hours post inoculation; mo-pi=Months post inoculation; ND=No Data, no sample available.

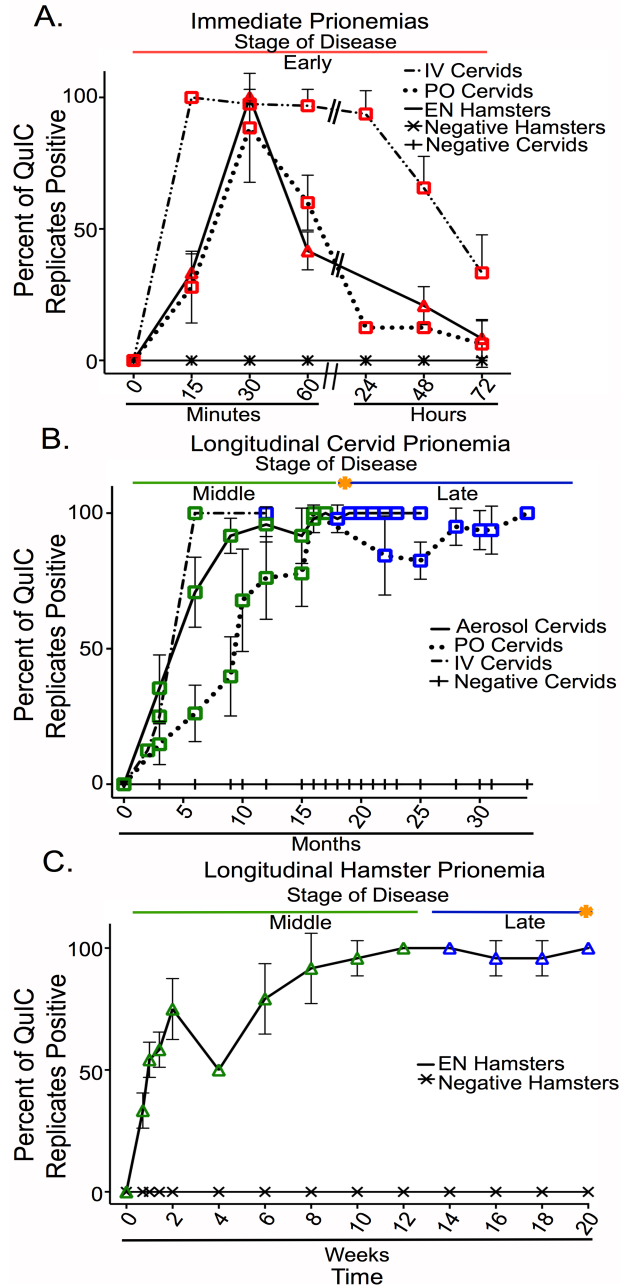


Figure 2.1 Immediate and longitudinal cervid and hamster prionemias.

Blood was collected from TSE-infected and TSE-naïve white-tailed deer, muntjac deer, and Syrian golden hamsters immediately following inoculation and throughout the course of disease until termination. All samples were run in 8 replicates via wbRT-QuIC and replicates within each inoculation route (i.e. IV, PO, aerosol, EN) were averaged together. Blood from cervids and hamsters was collected at 15, 30, and 60 min-pi as well as 24, 48, and 72 hpi (A). Longitudinal blood from IV-, aerosol- and PO-inoculated cervids was collected between 3 and 34 mo-pi (B). Longitudinal blood from hamsters was collected at 5, 7, and 10 dpi and 2-20 wpi (C). Early and middle stages of disease represent average asymptomatic disease prior to neurologic symptoms. Late stage represents the average occurrence of neuroinvasion. *: Average onset of symptomatic disease.

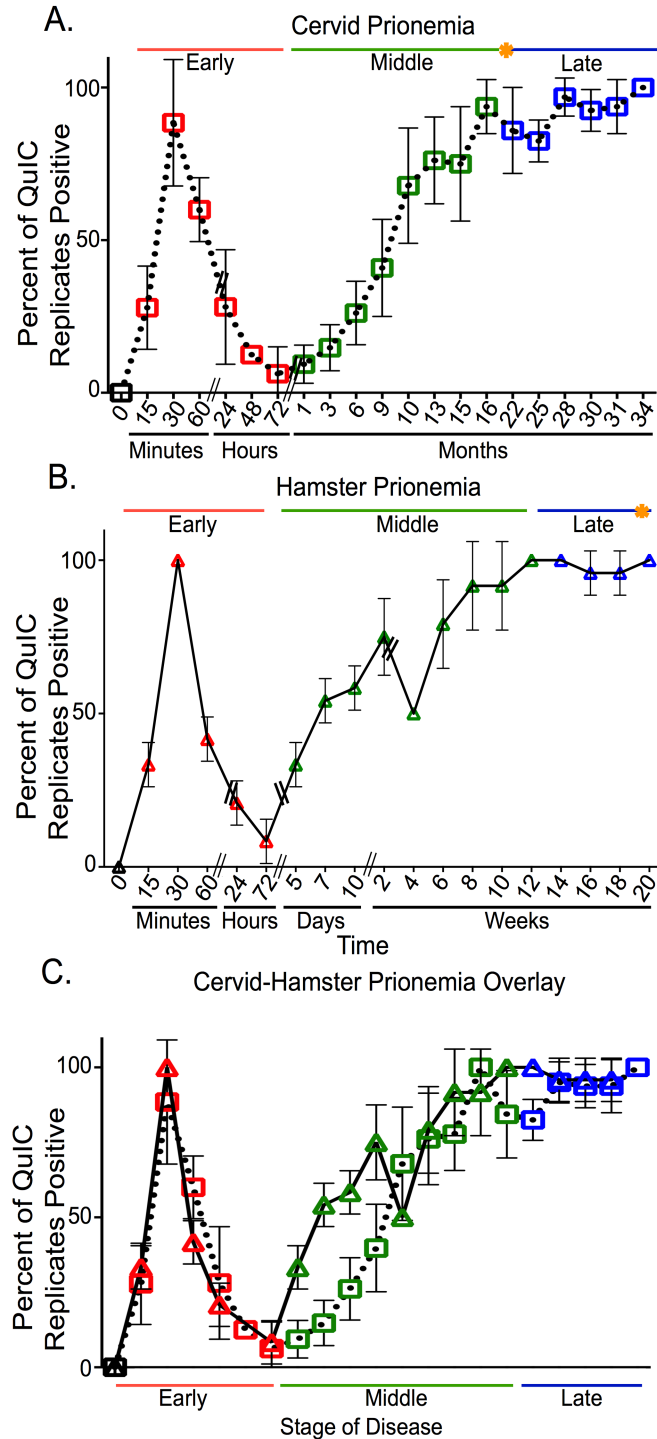


Figure 2.2 Complete cervid and hamster prionemias.

Data obtained from all blood collection time points—analyzed via wbRT-QuIC—were combined to visualize the complete course of prionemia throughout prion disease. Complete prionemia from orally-inoculated cervids (A) and complete hamster prionemia (B) are overlaid (C) to show consistency of prionemias throughout the course of disease. Early and middle stages of disease represent average asymptomatic disease prior to neurologic symptoms. Late stage represents the average occurrence of neuroinvasion. *: Average onset of symptomatic disease.

HY-TME prionemia:

Mucosal exposure: To assess prionemia in a second prion disease we investigated the longitudinal profile of HY-TME in hamsters. Blood harvested from HY-TME-infected hamsters, inoculated EN, at 15 min-pi had PrP^C-converting activity (33.33%), which was more consistently detected at 30 min-pi (100%) (Table 3; Fig.1A). Similar to CWD-exposed cervids, by 2% the disease course (72hpi) detectable converting activity had changed from 100% to 8.33% of replicates (p-value<0.0001), yet never dropped below assay detection levels (Table 3; Fig.1A). Nascent detectable PrP^C-converting activity was seen as early as 4% of the disease course (33.33% of replicates) and increased through 50% of disease (100% of replicates) where it remained through the course of disease (Table 3; Fig.1C; 2B, C) (data for hamsters 8 wpi-20 wpi previously shown in Elder, et al.[55]). PrP^C-converting activity was not detected in mock-infected hamsters.

These results demonstrate the ability of CWD and HY-TME prions to efficiently translocate across mucosal surfaces, enter the bloodstream, and replicate therein throughout the disease course.

Table 2.3 Complete hamster prionemia detection data

		Inoculation Route		
		Extranasal		
		Average positive replicates (8 per animal)	SD	<i>n</i>
Immediate Collections	0 min-pi	0.0	0.0	2
	15 min-pi	2.67	0.577	3
	30 min-pi	8.0	0.0	3
	60 min-pi	3.33	0.577	3
	24 hpi	1.67	0.577	3
	72 hpi	0.67	0.577	3
Longitudinal Collections	5 dpi	2.67	0.577	3
	7 dpi	4.33	0.577	3

10 dpi	4.67	0.577	3
2 wpi	6.0	1.0	3
4 wpi	4.0	0.0	3
6 wpi	6.67	0.577	3
8 wpi	7.33	1.15	3
10 wpi	7.67	0.577	3
12 wpi	8.0	0.0	3
14 wpi	8.0	0.0	3
16 wpi	7.67	0.577	3
18 wpi	7.67	0.577	3
20 wpi	8.0	0.0	3

SD=Standard Deviation; n =Number of animals sampled; min-pi=Minutes post inoculation; hpi=Hours post inoculation; dpi=Days post inoculation; wpi=Weeks post inoculation; N/A=Not applicable

PrP^{Sc}-conversion rates:

To determine if prion levels in the blood of infected animals truly plateaued, we analyzed the rate of PrP^{Sc} amyloid formation for each time point. We found that the reaction rate in whole blood samples emulated the percent of positive wbRT-QuIC replicates for all cohorts, demonstrating a plateau in the formation of blood-borne prion amyloid vs. assay detection limitations. For PO- and IV-inoculated cervids and EN-inoculated hamsters, the rate of amyloid formation declined substantially by 72 hpi (Fig.3). A subsequent increase in reaction rate was observed as early as 1 to 2 mo-pi in blood harvested from PO- and IV-inoculated cervids, respectively (Fig.3A). For all cervid inoculation groups, the rate of amyloid formation continued to increase until 12-14 mo-pi when the reaction rate reached a steady state (Fig.3A).

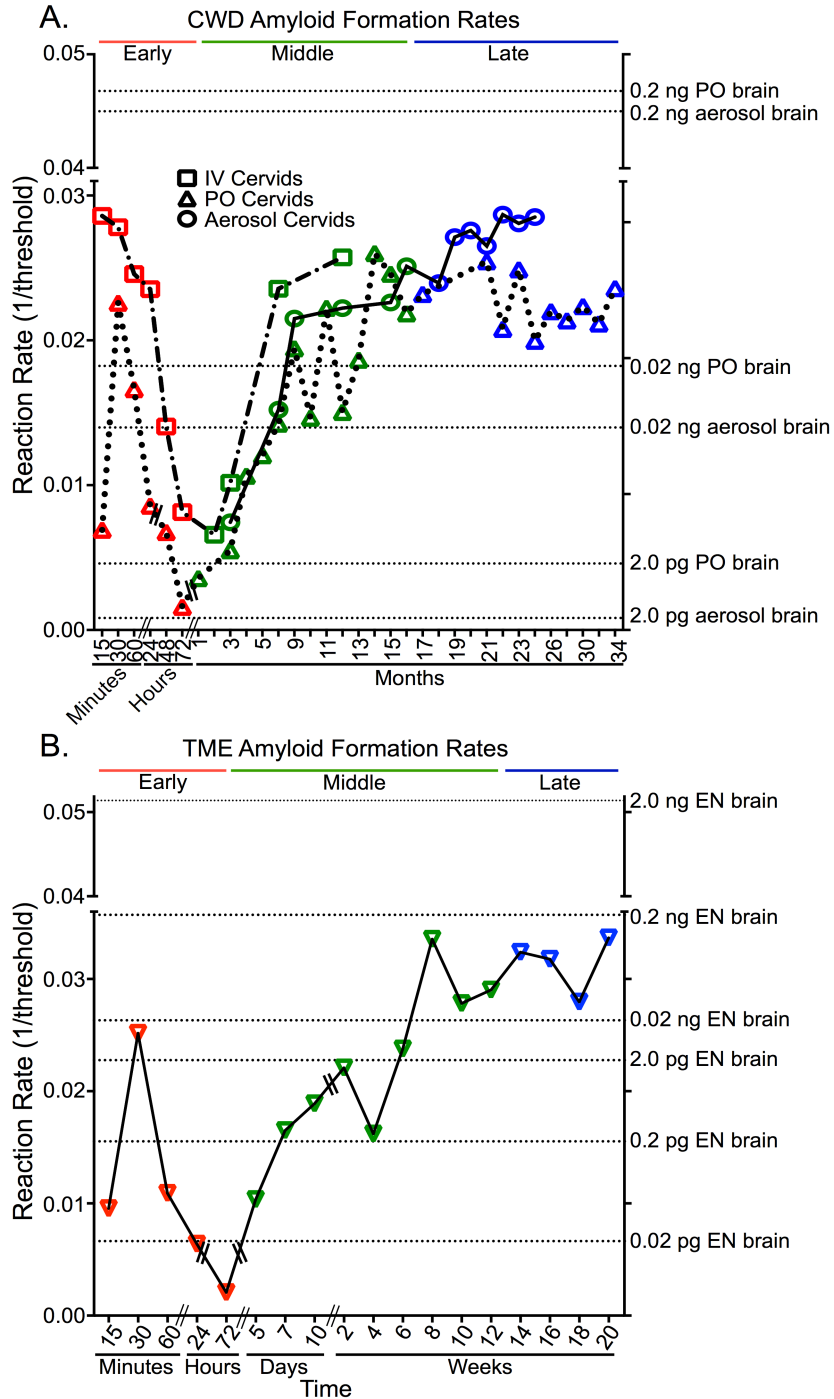


Figure 2.3 Rate of amyloid formation throughout CWD and TME prionemias.

The time to threshold (average of negative controls + 5 times the standard deviation) was calculated for data collected from all blood collection time points to determine the rate of amyloid formation. Amyloid formation rates for IV-, PO-, and aerosol-inoculated cervids (A) and EN-inoculated hamsters (B) were plotted over the entire course of infection. These were also compared to the rates of amyloid formation for various amounts of CWD- or TME-positive brain. Early and middle stages of disease represent average asymptomatic disease prior to neurologic symptoms. Late stage represents the average occurrence of neuroinvasion.

A similar initial spike and decline in the rate of amyloid formation was observed in whole blood collected from EN-inoculated hamsters as in cervid whole blood. This was followed by an increase in the amyloid formation rate as early as 5 dpi and continued to increase through 8 wpi when a steady state was reached (Fig.3B). While conversion rates fluctuated throughout the early and middle stages of disease (Fig.3), by late stage TSE disease all inoculation cohorts possessed rates equivalent to 0.2-0.02 ng of CWD-positive brain (Fig.3A) or HY-TME-positive brain (Fig.3B).

To quantitate PrP^{Sc} concentrations present in whole blood and to obtain a whole blood LD₅₀ we compared the reaction rate in whole blood collected from CWD- or HY-TME-infected animals to the amyloid conversion rate in CWD or HY-TME-infected brain as previously reported by Henderson et al.[58]. Our calculations suggest that 10 µl of whole blood collected from CWD-orally inoculated blood at 1 mo-pi contains 1.44 LD₅₀, while later in infection, 2 µl of whole blood contains 1.19 LD₅₀.

DISCUSSION

CWD and TME prionemia:

We set out to determine the temporal parameters of the hematogenous spread of prions, CWD in cervids and HY-TME in hamsters[55], using wbRT-QuIC *in vitro* analysis. Whole blood samples were longitudinally harvested from aerosol-, oral- and intravenous-infected cervids and extranasal-exposed hamsters. To date, three distinct phases have been identified after prion exposure: an infection phase, a replication phase, and a plateau phase[59; 60]. Our work reinforces the presence of these three phases, and further supports prion clearance shortly after

host TSE exposure[42; 59; 61]. In this study conversion-competent prions were rapidly detected in the blood of all prion-infected hosts regardless of inoculation route (Fig.1A, 2). Subsequent to this initial detection, which we believe to be point source inoculum, a near zero phase was observed (Fig.2). While we did not observe a total clearance of the inoculum, our results demonstrate the speed with which exogenous prions traverse mucosal linings and enter the circulatory system. From here, they are trafficked throughout the body, and subsequently appear to be mitigated by host immune clearance mechanisms. Eventually, there is a de novo development of amplification-competent prions that are spread in the blood of infected hosts and a subsequent plateau phase[59]. Our results provide evidence for this plateau phase; in late stage infection the rate of amyloid formation ceases to continue increasing and maintains a steady rate (Fig.3).

The early presence of amplification-competent prions in blood post transfusion infection is consistent with earlier work detecting radiolabeled PrP^{Sc} within minutes of IV-inoculation that persisted for several hours[42]. More interesting was the speed with which exogenous prions crossed mucosal surfaces and entered the circulatory system. These findings are consistent with previous results demonstrating the rapid transepithelial prion transport across nasal mucosa following inhalation[51]. Oral mucosa has been demonstrated to contain numerous permeable capillaries and a higher blood flow rate than many other tissues[62; 63], creating a permissive environment for prion entry into lymph and blood. Thus, prions introduced to mucosal surfaces (e.g. oral/nasal cavity) appear to gain immediate access to the blood, which precedes translocation to LRS tissues[45; 51]. The results of this work indicate that the mucosa that lines the nasal cavity and the gut is not an effective barrier to prion entry.

In this study, we noted a rapid, yet incomplete abatement of amplification-competent

blood-borne prions. While this may represent trafficking of the point source inoculum to peripheral tissues, it may also be the result of host-mediated clearance. Previous studies have demonstrated early PrP^{Sc} clearance[59], and macrophages, monocytes, and FDCs have been implicated[41; 59; 61]. Beringue and colleagues depleted splenic macrophage populations and reported a more rapid and diffuse PrP^{Sc} accumulation in macrophage-depleted animals than in animals with intact macrophage populations. The role of antigen presenting cells (APCs) in TSE dissemination or clearance was not addressed here, but earlier studies[41; 64] have noted the association of PrP^{Sc} with the fore-mentioned APC populations. A previous white-tailed deer bioassay study demonstrated that monocytes/macrophages are not associated with the transport of infectious prions[50]. It is plausible that these cell populations are involved in the host-mediated clearance of the prion agent within hours of gaining access to the circulatory system.

A nascent population of replication/amplification-competent prions was noted within days of inoculum abatement. It is possible that the initial inoculum was trafficked to lymphoid PrP^{Sc} amplification sites, or was amplified in cells within the circulatory system. The reemergence of detectable amplification-competent prions culminated in sustained higher levels of blood-borne prions over much of the disease course. A plateau phase consisting of high levels of PrP^{Sc} deposition and the presence of infectious prions has been documented for all prion diseases[18; 24; 29; 32; 55; 59; 65].

Rapid infiltration across mucosal surfaces to the blood has been previously identified in viral infections such as HIV, poliomyelitis, and measles[62; 66]. Once in the blood, a primary viremia is established followed by tissue-specific replication and viral overspill back into the blood[66]. This pattern is remarkably similar to that observed in these TSE blood samples. Despite the differences in TSEs and the infected-host species, both CWD and HY-TME

prionemias progress in similar timeframes (Fig.2C), indicating possible conserved TSE pathogenesis following mucosal exposure.

Implications of prions spread by blood:

While the exact mechanisms of how prions traffic to the brain following TSE exposure is unknown, there are two plausible routes whereby blood borne prions gain access to the central nervous system. In the first scenario, prions enter the body and are directly trafficked to lymphoid tissue and neuroinvasion occurs via the autonomic innervation of these tissues[44; 45]. Alternatively, prions gain entry to the brain through circumventricular organs (CVOs), or by breaching the blood-brain barrier[43; 44]. This possibility is supported by findings revealing that prions inoculated intraperitoneally into hamsters reached the brain within 2 days post inoculation[67] and the demonstration of PrP^{Sc} accumulation in CVOs regardless of inoculation route[43; 44; 68]. Our results demonstrate that prions quickly cross the mucosa into the circulatory system, making it plausible that prions are transported to the blood-brain barrier far earlier than previously suspected.

Knowing that conversion-competent prions are present in the blood within minutes of TSE exposure raises the possibility that peripheral tissues and organs may be exposed to prions much earlier than previously suspected. As blood circulates, it is highly probable that hematogenous prions are deposited in tissues early in infection. By extension, early infection and amplification of peripheral prions may play an important role in shedding of prions throughout the course of disease.

The results of this study also raise questions regarding the clearance of misfolded prions in infected animals. It is possible that an initial macrophage associated clearance accounts for the

sharp drop in PrP^C-converting activity observed. It is also feasible that a similar continued low-level clearance mechanism may help explain why, if prionemia is established early in infection, the disease course is so long. Clearly, additional studies are needed to address these questions.

The findings presented in this study provide the first temporal analysis of prionemia. The fact that prions efficiently traverse mucosal surfaces and are present in the blood throughout the entirety of infection—regardless of inoculation route—helps explain the wide-spread distribution of PrP^{Sc}, and provides insight for an alternate pathway of prion dissemination at the blood-brain barrier and shedding dynamics. Equally as interesting is the identification of a rapid hematogenous prion clearance. Unraveling the mechanisms associated with the abatement of amplification-competent prions from the blood will provide a better understanding of the pathogenesis and mitigation of prion diseases, and, by extension, of all protein misfolding diseases.

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CONCLUSION

While infectious prions are known to be present in many extraneural tissues, the lymphoreticular system (LRS) tissues have been identified to harbor the most prominent, and earliest, deposition. Variant Creutzfeldt-Jacob disease (vCJD) is known to have extensive LRS involvement and has also been detected in the blood of infected individuals. Certain animal prion diseases, specifically chronic wasting disease (CWD) and transmissible spongiform encephalopathy (TME), recapitulate human TSE pathogenesis, primarily in the LRS and blood. Importantly, infectious prions are present in the blood during both clinical and subclinical stages of disease and are transmitted efficiently through transfusion of incredibly small volumes of blood in the absence of overt clinical symptoms.

Previous to this work it was unknown if hematogenous prions are present in all infected hosts, if inoculation route affects the capability for prions to enter the blood, and when prions are present in blood. We have demonstrated that not only are blood-borne prions present in all animals infected with CWD or TME, but they are present in the blood throughout the entire course of disease, regardless of inoculation route. Here we demonstrate, for the first time, that prions cross mucosal surfaces and can be detected by *in vitro* methodology in blood within 15 minutes of TSE-exposure regardless of exposure route.

Early *in vitro* detection of TSEs in easily accessed fluids has long been considered a holy grail of prion research. An *in vitro* assay detecting blood-borne prions would provide: 1) blood and organ/tissue screening to prevent the further spread of human TSEs; 2) surveillance for wild and captive cervids to halt the spread of CWD to unaffected regions; 3) identification of potential reservoir species for natural TSEs; 4) better screening for cattle entering into food production

(currently only 1:40,000 are tested for BSE); 5) identification of possible compounds capable of prion inhibition that may aid in the development of vaccine and treatment options for TSEs; and 6) an entry point to aid the development of more efficient detection methods for other protein misfolding diseases (e.g. Alzheimer's disease, Parkinson's disease, and diabetes mellitus type II).

Early evidence for the dissemination of prions via hematogenous means is supported by studies demonstrating efficient transfusion transmission of contaminated blood products [1-9]. Previous attempts to analyze blood-borne prions have been fraught with difficulties due to inadequate *in vitro* detection specificity and sensitivity. The work of this thesis utilized longitudinally-collected blood samples from TSE-infected hosts and modifications made to the RT-QuIC assay (whole blood (wb) RT-QuIC) to assess amplification competent prions. We detected, for the first time, *in vitro* amplification competent hematogenous prions.

The works presented in this thesis demonstrate that: 1) prions can be consistently detected in blood from CWD-infected cervids and TME-infected hamsters; 2) prions cross mucosal surfaces quickly after TSE exposure and gain entrance to the circulatory system as early as 15 minutes post inoculation; and 3) the establishment of a *de novo* host-generated prionemia is observed soon after (CWD-infected cervids = 4 weeks; TME-infected hamsters = 5 days) post the detection of the initial inoculum.

The identification of prions in the blood of infected hosts throughout the entirety of disease poses interesting questions regarding the hematogenous transmission of TSEs from one susceptible host to the next. These findings support the association/involvement of blood-borne prions with prion shedding into the environment and the transmission of TSE infection via human blood transfusion or organ donation transmission. As infectious blood circulates throughout the host, it seems reasonable that PrP^{Sc} is deposited within peripheral tissues early in

infection, including those involved in fluid or excreta production (i.e. salivary glands, gastrointestinal (GI) tissues, and renal tissues). Infection of these tissues has been observed, as has the shedding of infectious prions in the fluids/excreta produced [2; 10; 11].

Human to human transmission has been previously observed, both through infected blood transfusion and organ transplant [12]. The four documented cases were the result of subclinical blood donors who later died of vCJD [5; 6; 13-15]. These occurrences have raised concern for a secondary outbreak of vCJD involving the transmission of blood-borne prions (as many as 1:2000 residents in the United Kingdom may be subclinical carriers of vCJD [16]). The fact that TSE-infected individuals may be subclinical for decades—or potentially never develop clinical disease—increases the probability of covert transmission to new susceptible hosts.

Our results suggest the presence of a primary prionemia—we propose consisting of the inoculated prions—and secondary prionemia (*de novo* host-synthesized prions). The presence of multiple blood phases has not been described for prions but has been observed in other pathogens, including viruses. In viral infections, the primary viremia occurs as the virus crosses mucosal barriers and enters the blood stream, while a secondary viremia is established following replication within tissues and newly synthesized virus entering the blood. The concept of multiple phases of prion infection is not novel with previous studies confirming 3 phases: infection, replication, and plateau [17; 18]. Our results suggest that within minutes of exposure the inoculum enters the circulatory system and is trafficked throughout the body. Upon replication within infected tissues the host-generated prions are likely shed back into the circulatory system and trafficked throughout the body.

The diminished detection of prions in the blood between the primary and secondary prionemia also indicates the possibility of a clearance mechanism for prions. Previous studies

have demonstrated that splenic macrophages are capable of clearing PrP^{Sc}, but not in its entirety, early in the infection stage of disease[17; 19]. A proposed clearance mechanism may explain our results indicating a drop in the detection of amplification competent hematogenous prions prior to *de novo* generation.

Blood-borne prions may be responsible for trafficking prions to various peripheral tissues, and may play a role in neuroinvasion as well. It has been hypothesized that prions first infect lymphoid tissues and are subsequently trafficked to the central nervous system (CNS) via autonomic nerves innervating these lymphoid tissues. However, a second hypothesis exists, one focusing on the use of blood with neuroinvasion occurring through the circumventricular organs (CVOs). In combination with the results reported in this thesis (i.e. the *in vitro* detection of amplification competent prions in blood within 15 minutes of PO-, EN, or IV-exposure), it is possible that neuroinvasion occurs via hematogenous infiltration at the CVOs as opposed to tissue deposition and transport via peripheral nerves.

We have shown that peripherally inoculated prions enter the blood early and persist throughout TSE disease establishing both a primary (inoculum) and secondary (*de novo* generation) prionemia. This work provides the first longitudinal *in vitro* temporal status of hematogenous prions providing a basis for continued investigations of the biological significance of blood-borne prions in TSE disease pathogenesis.

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ABBREVIATIONS

AAALAC: Association for Assessment and Accreditation of Laboratory Animal Care

AD: Alzheimer's disease

ALS: Amyotrophic lateral sclerosis

APC: Antigen presenting cell

BSE: Bovine spongiform encephalopathy

CJD (s,v,i): Creutzfeldt-Jacob disease (sporadic, variant, iatrogenic)

CNS: Central nervous system

CPDA: Citrate phosphate dextrose adenine

CVO: Circumventricular organ

CWD: Chronic wasting disease

Dpi: Days post inoculation

EDTA: Ethylenediaminetetraacetic acid

EN: Extranasal inoculation

FDC: Follicular dendritic cells

FFI: Fatal familial insomnia

GALT: Gut-associated lymphoid tissue

GI: Gastrointestinal tract

GSS: Gerstmann-Sträussler-Scheinker syndrome

HD: Huntington's disease

HRP: Horseradish peroxidase

IACUC: Institutional Animal Care and Use Committee

IB: Inclusion bodies

IC: Intracranial inoculation

IHC: Immunohistochemistry

IN: Intranasal inoculation

INF: Infected animal

IV: Intravenous inoculation

LB: Lysing buffer

LD₅₀: Median lethal dose

LRS: Lymphoreticular system

Min pi: Minutes post inoculation

MJ: Reeves' muntjac deer

Mo pi: Months post inoculation

NA: Not applicable

NaCl: Sodium chloride

NALT: Nasal-associated lymphoid tissue

NaPTA: Sodium phosphotungstic acid precipitation

ND: No data/not done

NIH: National Institutes of Health

PBS: Phosphate buffered saline

PD: Parkinson's disease

PK: Proteinase K

PLP: Paraformaldehyde-lysine-periodate

(s)PMCA: (serial) Protein misfolding cyclic amplification

PNS: Peripheral nervous system

PO: Oral inoculation

PrP^C: Cellular prion protein

PrP^{res}/PrP^{Sc}/PrP^D: Abnormal, misfolded, prion protein

PVDF: Polyvinylidene fluoride

rPrP: Recombinant prion protein

RT-QuIC: Real-time quaking-induced conversion

SD: Standard deviation

SD₅₀: Median seeding dose

SQ: Subcutaneous

TgCerPrP: Transgenic mouse expressing cervid prion protein

ThT: Thioflavin T

TME (HY or DY): Transmissible mink encephalopathy (Hyper or Drowsy strain)

TSE: Transmissible spongiform encephalopathy

UN: Uninfected animal

USDA: United States Department of Agriculture

Wb(o)RT-QuIC: Whole-blood (optimized) real-time quaking-induced conversion

Wpi: Weeks post inoculation

WTD: White-tailed deer