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Chronic wasting disease detection in the lymph nodes of free-ranging cervids by real time quaking-induced conversion

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Short running title: RT-QuIC detection of CWD in free-ranging deer

Abstract

Chronic wasting disease (CWD), a transmissible spongiform encephalopathy of deer, elk and moose, is the only prion disease affecting free-ranging animals. First identified in northern Colorado and southern Wyoming in 1967, new epidemic foci of the disease have since been

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identified in 20 additional states, as well as two Canadian provinces and the Republic of South Korea. Identification of CWD-affected animals currently requires post-mortem analysis of brain or lymphoid tissues using immunohistochemistry (IHC) or an enzyme linked immunosorbent assay (ELISA), with no practical way to evaluate potential strain types or investigate the epidemiology of existing or novel foci of disease. Using a standardized real time quaking-induced conversion (RT-QuIC) assay, a seeded amplification assay employing recombinant prion protein as a conversion substrate and Thioflavin T (ThT) as an amyloid-binding fluorophore, we blindly analyzed 1243 retropharyngeal lymph node samples from white-tailed deer, mule deer and moose, collected in the field from current or historic CWD-endemic areas. RT-QuIC results were then compared with those obtained by conventional IHC and ELISA, and amplification metrics using ThT and Thioflavin S examined in relation to clinical history of the sampled deer. The results indicate that RT-QuIC is useful in both for identifying CWD-infected animals and facilitating epidemiologic studies in CWD endemic and non-endemic areas.

Introduction

Chronic wasting disease (CWD) is an efficiently transmitted transmissible spongiform encephalopathy of cervids (e.g. deer, elk, and moose), and is the only known prion disease affecting free-ranging, non-domestic animals. As such, it is the only prion disease of animals whose control and eradication, through genotypic breeding schemes or herd reduction/depopulation efforts for example, is problematic. (1, 2) While the origins of CWD are uncertain, the disease has been present in wild cervid populations of northern Colorado and southern Wyoming for over 40 years (3, 4) and has now been identified in both captive and free-ranging cervids in 22 states, 2 Canadian provinces, and the Republic of Korea. (5) With intensified national and international surveillance efforts, CWD continues to be identified in areas previously thought to be free of infection, including recent discoveries in Iowa, Texas, and

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Pennsylvania. (6-8) The prevalence of CWD varies across North America, but can be as high as 30% in some areas of Colorado and approaching 80% in captive populations (9).

Determination of prevalence rates in a population is dependent on a sensitive and specific "gold standard" diagnostic assay. Immunohistochemistry (IHC) was, until recently, considered the gold standard diagnostic test for chronic wasting disease and other prion diseases of animals and man. In cervids, an ELISA assay was recently approved by the United States Department of Agriculture for primary diagnostic screening of field samples across the United States (10), though to date, an amplification-based assay, similar to PCR, for the detection of CWD (or other TSEs) has been elusive. The true sensitivity and specificity of IHC or ELISA in the detection of infected individuals is unknown, though it is generally acknowledged that the assay underestimates the level of prions in a given sample due to the necessity of a proteolytic pre-treatment step to abolish cellular PrP^C cross-reactivity (11-13). This limitation has led to increased interest in the development of assays that involve either amplification and detection of the protease-resistant prion protein (e.g. serial protein misfolding cyclic amplification, sPMCA) (14), fluorometric quantitation of seeded amplification activity (e.g. RT-QuIC) (15), or that otherwise avoid harsh proteolytic treatments (e.g. the conformation-dependent immunoassay, or CDI) (16).

One component of chronic wasting disease field surveillance that has required additional research is the ability to distinguish prion strains *in vitro*. Because prion infections are devoid of agent nucleic acids and a host immune response, conventional infectious disease strain-typing methods, e.g. nucleic acid sequencing or antibody neutralization studies, are not possible. Despite this hurdle, at least two strains of CWD have been reported in natural isolates – each yielding distinct pathological distribution in mouse bioassay and biochemical traits *in vitro*.(17-19) These strains may occasionally be found in the same individual, which, combined with the necessity of mouse bioassay, make epidemiologic studies difficult at best. Investigations into

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the unprecedented appearance of new epidemic foci across the US (e.g. southeastern Wisconsin in 2002, central New York State in 2005, and north-central Missouri in 2010) have relied on anecdotal information for the origin of CWD infection in these areas, with no additional insight into strain identities or source. The ability to distinguish strains *in vitro* has so far been limited to protease treatment or guanidine denaturation profiles, though fluorometric assays may hold promise in this arena and could be useful in epidemiological investigations. (20)

In the present study, we have applied a standardized RT-QuIC seeded amplification assay with two different fluorophores (thioflavin T and S – ThT, ThS) to blindly examine retropharyngeal lymph node (RLN) samples collected at necropsy from white-tailed and mule deer (*Odocoileus virginianus* and *O. hemionus*, n=1201), and moose (*Alces alces*, n=42) during routine CWD surveillance in Colorado, Illinois, Nebraska, New York, and Texas. We analyzed various aspects of amplification in positive animals: 1) time to threshold, 2) slope, and 3) peak fluorescence, and correlated our amplification results with several *a priori* variables – including age, sex, species, genotype, and harvest location. We hypothesized that RT-QuIC results would correlate to ELISA and IHC results reported by contributing state agencies, and that there would be amplification characteristics unique to either genotype or geographical regions of endemnicity. Our results demonstrate that RT-QuIC is comparable to conventional CWD detection assays in terms of sensitivity, and predict that seeded-amplification using various fluorophores may eventually prove to be a useful, rapid, and inexpensive tool for advanced epidemiological studies in ongoing and newly identified foci of chronic wasting disease.

Materials and Methods

Study population

The study areas included distinct geographic regions of Colorado, Illinois, Nebraska, New York, and Texas. Animals were harvested either during routine surveillance through the

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course of the 2010-2011 (NY, n=100, NE, n=280), or 2012 (TX, n=126) big game hunting seasons, as part of targeted CWD surveillance outside of big game seasons (2013, IL, n=695), or as road kill (2004-2010, CO, n=42). Lymph node samples were initially tested by either ELISA (New York State Department of Environmental Conservation/New York State Veterinary Diagnostic Laboratory, Nebraska Game and Parks Commission, University of Nebraska Veterinary Services Lab) or IHC (Texas Department of Wildlife and Parks/Texas State Veterinary Laboratory, Illinois Department of Natural Resources/Illinois Department of Agriculture, Colorado Division of Parks and Wildlife/Colorado State University Veterinary Diagnostic Laboratory), with results withheld until prion seeding assays were complete.

Tissue collection and processing:

Retropharyngeal lymph node samples (i.e. those specific tissues above) were collected during post-mortem examinations and submitted, frozen, to the Prion Research Center (PRC) at Colorado State University. Each sample was assigned a unique numerical designation and recorded to allow for blinded evaluation. Samples were initially prepared as a 2% (w/v) homogenate in RT-QuIC dilution buffer (phosphate-buffered saline, PBS, with 0.05% sodium dodecyl sulfate, SDS) using a BulletBlender ® (NextAdvance) with 0.5mm zirconium oxide beads and 1.5ml conical screw cap tubes. Samples were homogenized using three, 5-minute cycles of homogenization at a speed setting of 10, and were then kept at -80^{0}C until RT-QuIC analysis.

RT-QuIC procedure:

RT-QuIC assays were performed using a truncated form of the recombinant Syrian hamster PrP (SHrPrP residues 90-231) in pET41b and expressed and purified as previously described (21, 22). In brief, 1 liter cultures of lysogeny broth (LB) containing Auto Induction™ supplements (EMD Biosciences) were inoculated with SHrPrP expressing Rosetta strain *E. coli,*

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grown overnight, and harvested when optical density (OD, 600 nm) of ~3 was reached. Cells were lysed with Bug Buster™ reagent with supplemented Lysonase™ (EMD Biosciences) and inclusion bodies (IB) were harvested by centrifugation of the lysate at 15,000xg. IB pellets were washed twice and stored at -80°C until purification (typically 24 hours or less). IB pellets were solubilized in 8M guanidine hydrochloride (GuHCl) in 100mM $NaPO_4$ and 10mM Tris pH 8.0, clarified by centrifugation at 15,000xg for 15 minutes and added to Super Flow nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen) pre-equilibrated with denature buffer (6.0M GuHCl, 100mM $NaPO_4$, 10mM Tris pH 8.0). Denatured SHrPrP and Ni-NTA resin was incubated by rotating at room temperature for 45 minutes and then added to an XK fast protein liquid chromatography column (GE Healthcare). Refolding was achieved on column using a linear refolding gradient of denature buffer to refold buffer (100mM $NaPO_4$, 10mM Tris pH 8.0) over 340ml at 0.75ml/min. SHrPrP was eluted with a linear gradient of refold buffer to elution buffer (100mM NaPO4, 10mM Tris pH 8.0, 500mM imidazole pH 5.5) over 100ml at 2.0ml/min. Fractions were pooled and dialyzed against two changes of 4.0 liters of dialysis buffer (20mM $NaPO_4$ pH 5.5). Recovered SHrPrP was adjusted to a final concentration of ~0.5mg/ml.

Two percent RLN homogenates were diluted 1:100 in RT- QuIC dilution buffer, with 5µl of this resultant 0.02% homogenate added to 95µl of RT-QuIC reaction buffer (350mM NaCl, 10µM EDTA, 10µM thioflavin T – ThT - and 0.1mg/mL Syrian hamster $rPrP^C$), yielding a final lymph node homogenate concentration of $1x10^{-3}$ in RT-QuIC reaction buffer. These 100µl preparations were evaluated in parallel with both positive and negative control tissues in adjacent wells of a 96-well plate, along with unspiked controls spiked with 5µl RT-QuIC buffer alone. Control homogenates consisted of a pooled preparation of 6 CWD-positive white-tailed deer (CBP6) or tissue-matched negative controls collected in a CWD-negative area of New York State. Plates were then subjected to 96 cycles of shaking and incubation at 42^0C (approximately 24hrs), with cycles of 1 min shake (700rpm double orbital) and 1 min rest. ThT fluorescence readings (450nm excitation and 480nm emission, bottom read, 20 flashes per well)

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were taken following each 15 minute cycle using a gain setting of 1200. Positive samples were defined as those that crossed a threshold of fluorescence; a threshold determined by the average fluorescence of the three tissue-matched negative controls over the course of the experiment plus five standard deviations. Time to positivity was defined as the time at which a sample fluorescence emission crossed the threshold (C_t). Time to positive fluorescence of test samples was then compared to that of positive control tissue, arriving at a value typically between 0-1; samples with earlier times to threshold fluorescence thus had values closer to or greater than 1. The slope of the amplification curve was determined as the increase in relative fluorescence over time. These analyses were performed using MARS analytical software.

Positive samples were separately evaluated, in triplicate in three separate experiments, using two different protocols: the first using ThT, as described above, the second with thioflavin S (ThS, 10μm) in place of ThT. Thioflavin S fluorescence readings (480nm excitation and 510nm emission, bottom read, 20 flashes per well) were taken following each 15 minute cycle using a gain setting of 1400. Criteria for identification of seeding activity in both protocols were again performed as described above for ThT. Values for time to positive threshold, slope, and fluorescence plateaus were averaged across the 9 replicates for each sample and each fluorophore.

Cervid PRNP PCR amplification and sequence analysis:

DNA was extracted from frozen, CWD-positive RLNs using a commercial kit (Qiagen) following the manufacturer's instructions. Consensus primer pairs specific for amplification of *PRNP* in mule deer and white-tailed deer have been previously described by O'Rourke and colleagues (23). PRNP sequences were amplified using HotStart DNA polymerase (Qiagen) with forward primer 223 5′-acaccctctttattttgcag-3′ and reverse primer 224 5′-agaagataatgaaaacaggaag-3′, which yielded an approximately 830bp product. PCR reaction conditions were as follows: 95□°C for 5 min, followed by 35 cycles of denaturation (95□°C, 60

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s), annealing (54□°C, 60 s) and extension (72□°C, 60 s) followed by an extension cycle (72□°C, 7 min) under standard buffer conditions with 2.5 mM $MgCl_2$ (Qiagen). PCR products were analyzed on 1.5□% agarose EZ-vision-stained gels.

PCR products were then purified using a commercial kit (Qiagen) to remove unincorporated dNTPs and primers, then bidirectionally sequenced using forward primer 223 and reverse primer 224 (GeneWiz Inc., South Plainfield, NJ). Chromatogram data were aligned using the CLC Main Workbench 6.8.4 software. All sequences were individually analyzed for conflicts and secondary peaks in order to create all necessary contigs and associated consensus sequences. DNA and amino acid sequences were aligned using ClustalW (Codons) in Mega 5.2 to determine amino acid polymorphisms, focusing on amino acid residues 95 and 96 (white-tailed deer) and 225 (mule deer).

Analysis of RT-QuIC metrics:

Our analyses had two major foci: (1) to determine if there was a relationship between ELISA scores and RT-QuIC metrics (ThT and ThS score, amplitude and slope) for CWD positive deer, and (2) to evaluate what were the best predictors (location/state, species, sex, age, and PrP genotype at amino acid position 96) of RT-QuIC metrics for CWD positive mule deer and whitetail deer. All analyses were conducted using the program R (www.r-project.org), using the stats package.

To determine if there was a relationship between ELISA scores and RT-QuIC metrics, we performed Spearman correlations. ELISA scores were only available for deer from Nebraska, and we evaluated the relationships for all deer and for mule deer and whitetail deer independently.

To evaluate how well the RT-QuIC metrics were predicted by the harvest location, deer species, sex, age and PrP genotype at position 96, we employed an Information Theoretic approach (24), whereby all single predictor variables were evaluated using linear regression,

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based on a Gaussian distribution, and ranked based on Akaike Information Criterion corrected for small sample size (AICc, **Supplementary Table 1**) (24). This approach is of value because it enables determination of the most parsimonious model or set of models to explain RT-QuIC metric scores, and also calculation of Variable Importance weights to determine the relative importance of one predictor variable over others (24). Because Information Theory departs from frequentist based statistical approaches, which are dependent on *P*-values, we also calculated the coefficient of variation (r^2) so that the relative fit of models to the data could be assessed.

Results

Conventional detection of PrP^{res} in RLN tissue:

Retropharyngeal lymph nodes were analyzed by referring state agencies using either conventional ELISA (NY, NE), or IHC (CO, IL, TX). The Colorado State University Veterinary Diagnostic Laboratory did not detect CWD infection in 42 moose from Colorado, while the University of Nebraska Veterinary Diagnostic Center identified 5 mule deer and 5 white-tailed deer as positive for CWD by ELISA. Ages ranged from 1-3 years, with both sexes and only homozygous 95Q/Q and 96G/G (white-tailed deer), and 225S/S (mule deer) animals represented. ELISA scores ranged from 2.961-3.292. The Illinois Department of Agriculture identified twelve of 695 white-tailed deer samples as CWD-positive by IHC. Ages ranged from <1yr-3yrs of age, with both sexes and both 96G/G and 96G/S genotypes represented; all animals were homozygous for glycine at amino acid position 95. The Texas Veterinary Services Laboratory identified one of 126 mule deer samples as CWD-positive by IHC (225S/S), while the New York State Veterinary Diagnostic Laboratory did not identify any CWD-positive animals among the 100 white-tailed deer samples submitted to the PRC for analysis. (**Table 1**)

RT-QuIC analysis of RLN tissues:

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RT-QuIC analysis of RLN samples from Illinois revealed seeded amplification in 12/695 samples, corresponding to IHC-positive samples. Analysis of samples from Nebraska showed seeded amplification in 10/280 samples corresponding to ELISA positive lymph nodes, while 1/126 samples submitted by the Texas Department of Parks and Wildlife demonstrated seeded amplification in RT-QuIC; RLN from this deer was also positive by IHC. Forty-two RLN samples from moose in Colorado and 100 white-tailed deer in NY remained negative by RT-QuIC. **(Figures 1 & 2)** RT-QuIC positivity correlated 100% with positivity by IHC and ELISA (i.e. 100% sensitivity and specificity), with amplification scores ranging from 0.479-1.06 using ThT, and 0.521-1.16 using ThS. RT-QuIC results for CWD-positive animals are summarized in **Table 1**.

Correlation between RT-QuIC amplification analyses and clinical variables:

Comparing ELISA to RT-QuIC, the direction of relationships between ELISA scores and RT-QuIC metrics were generally similar among deer species (**Table 2**). The notable exception to this was ThT slope – positive and negative slopes for mule deer and whitetail deer respectively. However, correlation values indicate that the positive relationship observed for mule deer was only weakly supported, whereas the negative slope between ELISA score and ThT slope was a moderately strong trend. Overall, and for white tail deer, there was a significant positive correlation between ELISA score and ThS slope.

When evaluating RT-QuIC metrics as predictors of *a priori* variables, there was generally one predictor variable that was distinctly a better/more important (variable importance weight > 0.4) predictor than the rest (Figure 3, Table 3). This was particularly distinct for ThT slope, ThS score, ThS amplitude and ThS slope (Figure 4c,d,e,f). However, there was model uncertainty for the best predictor of ThT score and ThT amplitude (Figure 3a,b). For RT-QuIC metrics with one predictor variable better than the rest (variable importance weight > 0.4), we plotted that association with the RT-QuIC metric (Figure 4). ThT slope and ThS amplitude exhibited positive

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and negative relationships with deer age, while female deer tended to have higher and less variable ThS scores. Interestingly, ThS slope was higher in the limited number of individual deer with 96G/S genotype present.

Discussion

Amplification-based assays, long used in a clinical setting for the detection of viral and bacterial agents in clinical samples, have to date not been available for use in post-mortem screening for transmissible spongiform encephalopathies. The slow evolution of prion amplification assays, which take advantage of the propensity for the abnormal PrP^{res} isoform to convert mammalian – and more recently recombinant PrP^{C} – in vitro, has made it increasingly practical to employ these approaches in a clinical setting. The real time quaking-induced conversion (RT-QuIC) seeded amplification assay offers the additional advantage of avoiding the proteolytic or acidic pretreatments commonly required for both conventional TSE detection assays (e.g. immunohistochemistry and ELISA) and serial protein misfolding cyclic amplification (sPMCA). RT-QuIC has been reported to amplify PrP^{res} seed present in brain dilutions in the femtogram range, comparable to bioassay (22, 25); however in the present manuscript our goal was to compare seeded amplification directly to conventional IHC and ELISA on samples collected post-mortem.

Our findings, through blinded analysis of over 1200 field samples collected from various cervid species across the United States, demonstrate that RT-QuIC is capable of accurately identifying IHC- and ELISA-positive retropharyngeal lymph nodes. With continued development of the RT-QuIC assay, it may be possible to identify subclinically positive, TSE affected individuals not positive by conventional detection systems, which may represent a significant number of animals in CWD endemic areas. It remains to be shown whether RT-QuIC could be used for antemortem detection of CWD infection, using rectal biopsies or other clinical samples available antemortem (e.g. blood or CSF). (21, 26, 27)

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Apart from enhanced sensitivity, conventional amplification assays for viral and bacterial pathogens offer a second distinct advantage – the ability to identify pathogen-derived nucleic acid sequences or specific antibody responses, facilitating epidemiologic investigations. It is commonly accepted that infectious prions lack both a nucleic acid component and a specific host immune response, yet still exhibit distinct strain properties; therefore alternative methodologies for identifying TSE strains *in vitro* are necessary. A number of fluorophores have been shown to bind prion aggregates; indeed, this finding has been incorporated into the RT-QuIC assay, providing visual evidence of seeded amplification through cumulated binding of one of these fluorophores – Thioflavin T. Little is known about how or where in the prion protein structure this binding may occur, though strain discrimination has been reported using luminescent conjugated polymers (LCPs), fluorophores that emit conformation-dependent fluorescence spectra. (20) Our analysis of CWD-positive lymph nodes using two conventional fluorophores revealed that some components of the RT-QuIC analysis scheme, including Thioflavin S score, amplitude, and slope, may be predictors of a CWD-positive cervid's background or possibly CWD strain traits. No definitive evidence of geographic grouping was observed across ThT or ThS metrics, though it is possible that incorporation of LCPs into the RT-QuIC assay may allow for a more precise discrimination of clinical TSE isolates and eventual strain correlation.

In summary, we report the first deployment of an amplification-based assay for the detection of CWD in cervid field cases. Blinded RT-QuIC analysis yielded results correlating directly to those of conventional IHC and ELISA. Further work is needed to assess whether RT-QuIC analysis may or may not contribute to CWD strain distinction, antemortem detection, and advanced epidemiological studies.

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400 **Table 1. Characteristics of CWD positive deer samples, as determined by RT-QuIC.** States are Nebraska (NE), Illinois (IL) and
401 Texas (TX). Species are mule deer (MD) and whitetail deer (WTD). Deer age is in years, with individuals <1 year old assigned 0.5
402 for analyses. Heterozygosities indicated in bold. NA: data was not available for that particular sample.

Animal							Amino Acid			ThT			ThS		
ID	State	County	Species	Sex	Age	ELISA	95	96	225	score	amp	slope	score	amp	slope
1059	NE	Box Butte	MD	F	3	3.143	QQ	GG	SS	0.885	10818	1.514	1.080	6584	0.429
1088	NE	Sioux	MD	M	3	3.079	QQ	GG	SS	0.445	6243	1.878	0.619	6730	0.376
1127	NE	Phelps	WTD	F	2	3.292	QQ	GG	SS	0.768	10329	1.722	0.964	6778	0.407
1128	NE	Buffalo	WTD	M	1	3.292	QQ	GG	SS	0.702	10047	1.017	0.865	6973	0.335
1154	NE	Furnas	WTD	M	2	2.961	QQ	GG	SS	0.506	8594	1.797	0.589	6895	0.274
1187	NE	Sioux	MD	M	2	3.151	QQ	GG	SS	0.844	11146	1.716	1.025	7162	0.434
1218	NE	Webster	WTD	M	2	3.104	QQ	GG	SS	1.060	11409	1.704	1.037	5879	0.322
1254	NE	Custer	WTD	M	3	2.966	QQ	GG	SS	1.025	12314	1.753	0.996	5800	0.285
1267	NE	Scottsbluff	MD	M	1	3.11	QQ	GG	SS	0.811	10293	2.250	0.718	5831	0.283
1268	NE	Sioux	MD	F	NA	2.983	QQ	GG	SS	0.818	11170	1.228	0.826	5890	0.289
1344	TX	El Paso	MD	M	4.5	NA	QQ	GG	SS	0.666	10233	2.691	0.966	5126	0.375
1468	IL	Kane	WTD	M	2	NA	QQ	GG	SS	0.691	8561	2.247	0.684	5376	0.452
1475	IL	McHenry	WTD	M	3	NA	QQ	GG	SS	0.676	8805	3.167	0.659	5013	0.610
1543	IL	Stephenson	WTD	F	2	NA	QQ	GG	SS	0.751	11172	0.861	0.761	6928	0.363
1563	IL	Boone	WTD	F	<1	NA	QQ	GG	SS	0.479	7725	0.810	0.984	5886	0.378
1606	IL	Dekalb	WTD	F	2	NA	QQ	**GS**	SS	0.846	11165	0.901	0.929	6277	0.357
1617	IL	Dekalb	WTD	F	1	NA	QQ	GG	SS	0.818	11187	0.768	0.955	6191	0.340
1761	IL	McHenry	WTD	M	1	NA	QQ	GG	SS	0.979	11999	0.846	1.164	5765	0.400
1771	IL	Grundy	WTD	F	2	NA	QQ	GG	SS	0.784	11086	1.179	1.005	6288	0.340
1801	IL	Kane	WTD	M	1	NA	QQ	GG	SS	0.879	6784	0.773	0.766	7243	0.365
1912	IL	Ogle	WTD	F	<1	NA	QQ	GG	SS	1.022	10855	2.771	0.912	7636	0.371
1918	IL	Dekalb	WTD	M	1	NA	QQ	GG	SS	0.824	9278	1.386	0.725	7139	0.373
1923	IL	Ogle	WTD	M	3	NA	QQ	**GS**	SS	0.848	10352	1.516	0.521	5684	0.875

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Table 2. Spearman correlations between ELISA scores and QuIC metrics for CWD positive deer. Significant results in bold. Notably, for individual deer species, ρ values indicate the strength of relationships, but the statistical power to detect significant relationships is lowered owing to the reduced sample size in these cases.

	All deer (n=10)		Mule deer (n=5)		Whitetail deer (n=5)	
	ρ	p	ρ	p	ρ	p
ThT score	-0.024	0.947	0.600	0.350	0.154	0.805
ThT amplitude	-0.116	0.751	0.000	1.000	0.051	0.935
ThT slope	-0.377	0.283	0.200	0.783	-0.821	0.089
ThS score	0.413	0.235	0.600	0.350	0.154	0.805
ThS amplitude	0.456	0.185	0.500	0.450	0.308	0.614
ThS slope	0.699	**0.024**	0.700	0.233	0.975	**0.005**

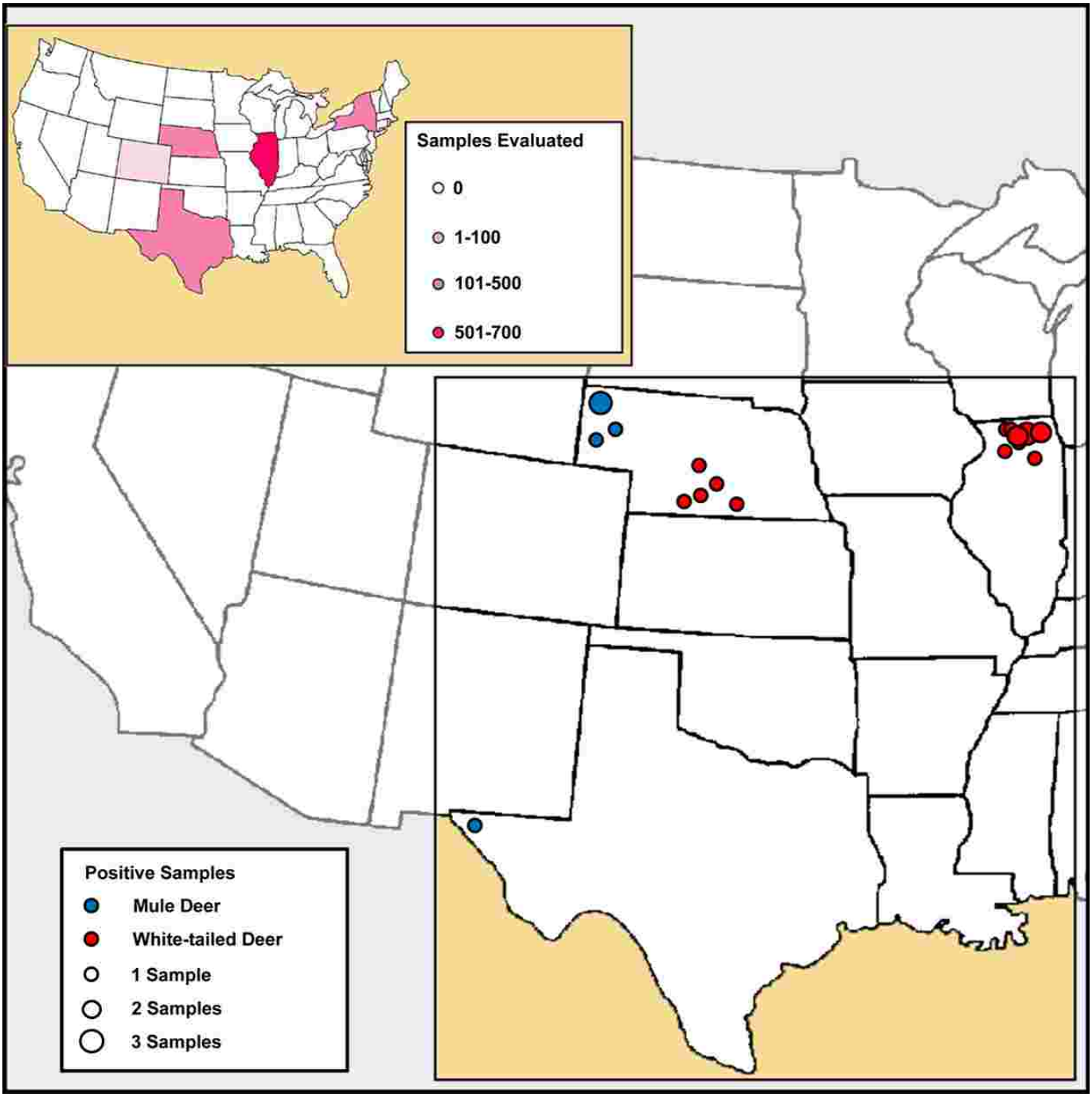
Figure 1. Summary of retropharyngeal lymph node samples evaluated and positive sample locations. Samples included 100 white-tailed deer lymph nodes from New York State, 695 white-tailed deer lymph nodes from Illinois, 280 white-tailed and mule deer lymph nodes from Nebraska, 126 mule deer lymph nodes from Texas, and 42 moose lymph nodes from Colorado. Of 1243 samples evaluated, 11 RT-QuIC positive deer were identified in Illinois, 10 positive in Nebraska, and a single positive deer in Texas.

Figure 2. RT-QuIC results from CWD-positive deer. Samples positive during initial screening were reanalyzed in triplicate in three separate experiments using either Thioflavin T or Thioflavin S (ThT or ThS, respectively). Positive controls (CBP6) as well as multiple negative controls (CWD-negative lymph nodes and untreated recombinant PrP) were included on each experimental plate. The threshold for amplification (orange dotted line) was determined by averaging the relative fluorescent units (RFUs) of negative control samples over the course of the experiment and adding five standard deviations. Seeded amplification is demonstrated by

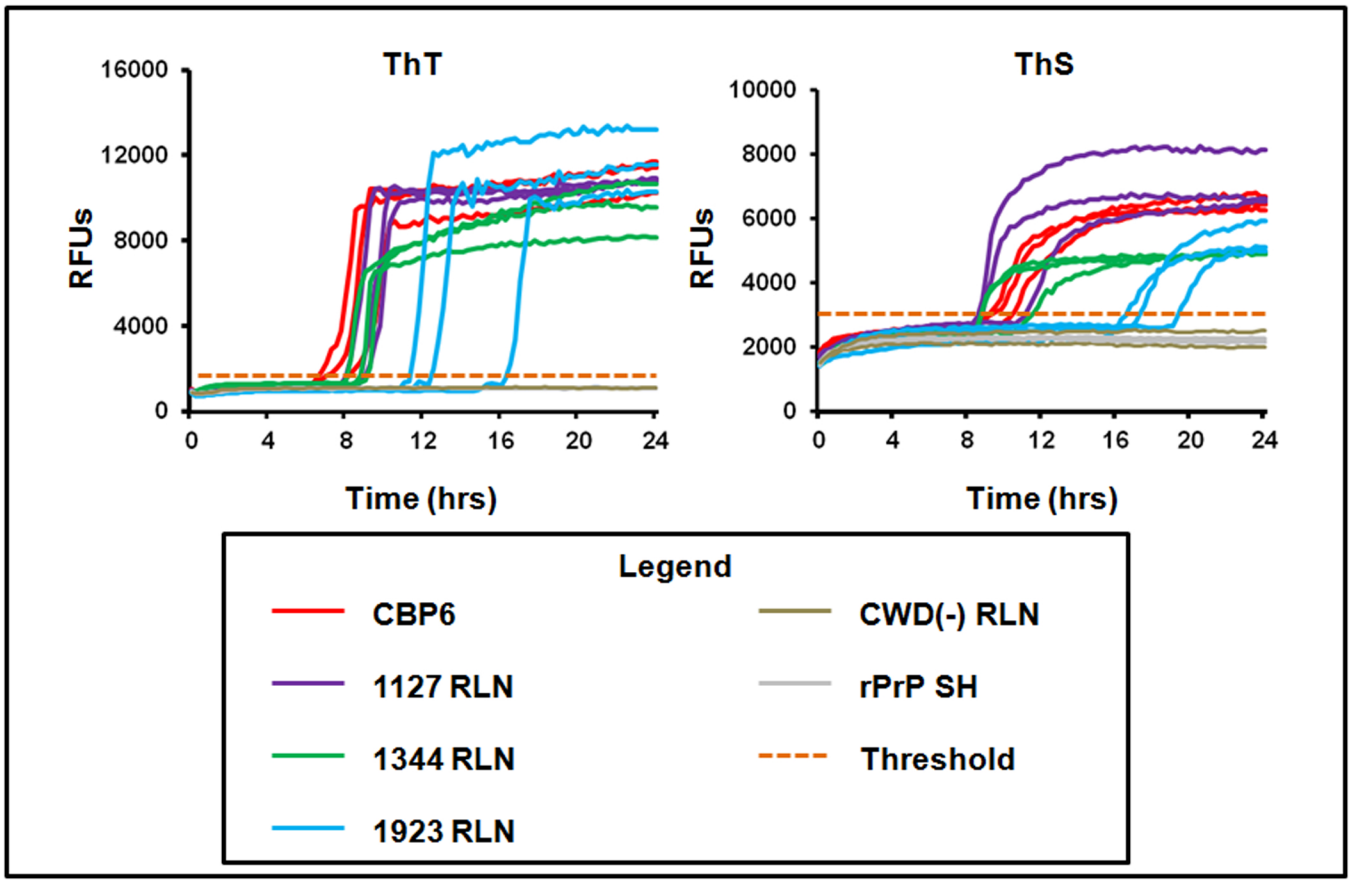
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424 increases in ThT and ThS fluorescence over time in the positive control sample as well as each
425 of three positive lymph nodes from study deer; negative controls do not show seeded
426 amplification.
427
428 **Figure 3. Variable importance weights of location, species, sex, age and amino acid**
429 **predictors of with QuIC metrics**. (a) ThT score, (b) ThT amplitude, (c) ThT slope, (d) ThS
430 score, (e) ThS amplitude, and (f) ThS slope. Where there was clearly a 'best' predictor variable,
431 variable importance weights were > 0.4, denoted by vertical line. Of the correlates analyzed,
432 likely predictors of "Age" included ThT slope and ThS amplitude, while ThS score correlated
433 with "Sex" and ThS slope seemed to be a good predictor of amino acid 96 identity. Full model
434 comparison tables in Supplementary Table 1.
435
436 **Figure 4. Distribution of QuIC metric data associated with the best predictor variable in**
437 **each case** (see Figure 3). Coefficient of variation given on each figure. These results show a
438 positive correlation between ThS slope and cervid PrP amino acid position 96 identity. See
439 Supplementary Table 1 for full model comparison table.

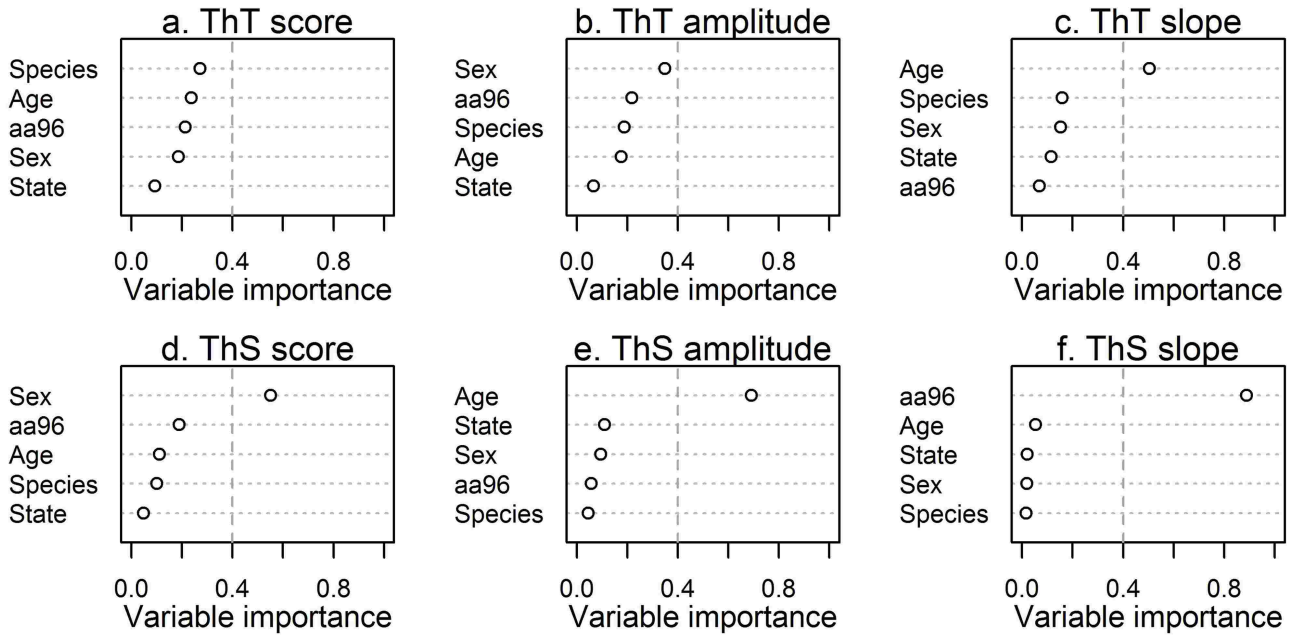
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