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Comparative study of immunoassays, a microelectromechanical systems-based biosensor, and RT-QulC for the diagnosis of chronic wasting disease in white-tailed deer

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Abstract

Background Chronic wasting disease (CWD) is a fatal transmissible spongiform encephalopathy in cervids. The disease is caused by a pathogenic prion, namely PrP^{Sc}. Currently, diagnosis of CWD relies on IHC detection of PrP^{Sc} in the obex or retropharyngeal lymph nodes (RPLN) or ELISA screening of obex and RPLN followed by IHC confirmation of positive results. In this study, we assessed the performance characteristics of two immunoassays: CWD Ag-ELISA and TeSeE ELISA, RT-QulC, and MEMS biosensor via testing 30 CWD+ and 30 CWD- white-tailed deer RPLN samples.

Results Both CWD Ag-ELISA and TeSeE ELISA correctly identified all CWD+ and CWD- samples. A greater intra-assay coefficient of variation (CV) in S/P ratios was observed for the TeSeE ELISA (16.52%), compared to CWD Ag-ELISA (9.49%). However, the high CV did not affect the qualitative results of triplicate assays when the corresponding manufacturer's cutoff was used. The MEMS biosensor not only correctly identified all CWD+ and CWD- RPLN samples, but also demonstrated a 100% detection rate for all CWD+ samples at dilutions from 10⁻⁰ to 10⁻³. Evaluation of RT-QulC indicated that the rate of false negative reactions decreased from 21.98% at 10⁻² dilution to 0% at 10⁻⁴ and 10⁻⁵ dilutions; and the rate of false positive reactions reduced from 56.42% at 10⁻² dilution to 8.89% and 2.22% at 10⁻⁴ and 10⁻⁵ dilutions, respectively. Based on a stringent threshold of 2 x the first 10 fluorescent readings of each well and a final cutoff of 2/3 positive reactions for each sample, RT-QulC correctly identified all positive and negative samples at 10⁻⁴ and 10⁻⁵ dilutions. Both MEMS biosensor and RT-QulC achieved 100% sensitivity and 100% specificity under the experimental conditions described in this study.

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Conclusions The two immunoassays (CWD Ag-ELISA and TeSeE ELISA) performed comparably on white-tailed deer RPLN samples. MEMS biosensor is a reliable portable tool for CWD diagnosis and RT-QuIC can be used for routine testing of CWD if appropriate testing parameters and interpretive criteria are applied.

Keywords CWD, deer, RPLN, ELISA, S/P value, IHC, MEMS biosensor, Dielectrophoresis, Rapid detection, RT-QuIC, Sensitivity, Specificity

Background

Chronic wasting disease (CWD) is a fatal transmissible spongiform encephalopathy (TSE) in cervids, such as mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), elk (*Cervus canadensis*), sika deer (*Cervus nippon*), and moose (*Alces alces*) [1–3]. The causative agent of the disease is a pathogenic prion, designated as PrP^{Sc} [4]. Although the emergence of PrP^{Sc} remains to be understood, it is known that PrP^{Sc} induces conversion of normal cellular prion protein (PrP^C) to PrP^{Sc}, a misfolded infectious protein that is resistant to proteases and to commonly used disinfection procedures [5–7]. Accumulation of PrP^{Sc} aggregates in brain tissue results in a neuropathology characterized by spongiform degeneration, neuronal loss, and glial activation which leads to clinical manifestations, including progressive loss of body condition, tremor, nervousness, excessive salivation, and death [8–11]. Since the first report of CWD in the 1960s, the disease has spread to free-ranging cervids in 32 U.S. states and four Canadian provinces and in captive cervid facilities in 18 U.S. states and three Canadian provinces [12, 13]. The transmission of CWD involves direct contact with cervid tissues and excreta containing the prion and indirect contact with environmental fomites contaminated by PrP^{Sc} [2, 14–16]. To detect CWD as early as possible in new locations and monitor changes in disease occurrence over time, the Missouri Department of Conservation has implemented stringent CWD control measures, including the CWD hunter harvest initiative, CWD management zone, and CWD surveillance testing (<https://mdc.mo.gov/hunting-trapping/species/deer/chronic-wasting-disease/cwd-surveillance>).

Several techniques have been used to detect CWD in cervid samples including immunohistochemistry (IHC), antigen capture enzyme linked immunosorbent assay (ELISA) or enzyme immunoassay (EIA), protein misfolding cyclic amplification (PMCA), and real-time quaking-induced conversion (RT-QuIC) [17–21]. Currently, IHC detection of PrP^{Sc} amyloid fibrils in the obex of the brain stem or the retropharyngeal lymph node (RPLN) is considered the gold standard and required by the United States Department of Agriculture (USDA) for the Voluntary CWD Certification Program [22]. ELISA is utilized routinely by veterinary laboratories to screen CWD prion in wild and free-range cervids, ELISA positive results are confirmed by IHC. Both IHC and ELISA have high diagnostic sensitivity and specificity for obex and RPLN

samples collected from animals in advanced stages of disease [23–27]. CWD ELISA (or EIA) is based on the sandwich ELISA principle which involves immobilizing PrP^{Sc}-specific ligand to the wells of an ELISA plate, applying an aliquot of Obex or RPLN tissue homogenate, and adding an enzyme-labeled antibody against PrP^{Sc} to form Ab (ligand)-Ag-Ab-enzyme complex, and then an appropriate substrate for color development as an indicator of positive reaction [28]. RT-QuIC takes advantage of the ability of pathogenic prion to convert a recombinant PrP as an assay substrate to a misfolded, aggregated form of rPrP which can be detected by measuring the fluorescence of Thioflavin T incorporated into the newly formed misfolded protein [25, 29, 30]. The formation of PrP^{Sc} aggregates during RT-QuIC reaction is monitored in real time by measuring thioflavin-T incorporation into amyloid fibrils [25, 29]. RT-QuIC, a technology that is suitable for high throughput testing and free of generating infectious prions, can be optimized to achieve high accuracy and consistency for CWD diagnosis, especially with samples collected during early presymptomatic stages of CWD [21, 31, 32]. The impedance-based micro-electro-mechanical systems (MEMS) biosensors have been used to detect various pathogens and toxins in clinical and food samples [33, 34]. Recently, we have demonstrated that a MEMS biosensor is able to detect PrP^{Sc} in cervid blood and tissue samples as well as the misfolded proteins in the ELISA positive control [35]. The CWD MEMS biosensor measures the impedance change induced by the binding monoclonal antibody immobilized on the detection electrodes to PrP^{Sc} in testing sample [35].

In this study, we evaluated the performance of immunoassays, MEMS biosensor, and RT-QuIC for the detection of PrP^{Sc} in hunter-harvested white-tailed deer RPLN samples. Our goal is to develop and validate new technologies for CWD diagnosis.

Materials and methods

Testing samples

As part of Missouri CWD surveillance program, white-tailed deer retropharyngeal lymph nodes (RPLN) were collected from hunter-harvested dead deer and submitted by the Missouri Department of Conservation (MDC) to the University of Missouri Veterinary Medical Diagnostic Laboratory (VMDL) for CWD diagnosis. Sample collection and submission protocols were approved by MDC Science Branch leadership committee. The samples

were initially tested using an antigen-capture enzyme immunoassay according to the manufacturer's instruction (HerdChek CWD Ag-ELISA, IDEXX). ELISA positive results were confirmed by IHC according to the National Veterinary Services Laboratories' protocol (NVSL-SOP-0878). ELISA negative results were accepted as negative for CWD prion without further analysis by IHC. After diagnostic testing, the samples became VMDL property per VMDL-I-002, and were stored at -80°C for additional scientific investigations. From the sample repository, 30 CWD+RPLN samples and 30 CWD- RPLN samples were retrieved for the present comparative study of TeSeE ELISA (Bio-Rad Laboratories), CWD Ag-ELISA (IDEXX Laboratories), MEMS biosensor, and RT-QuIC. This research utilized diagnostic samples to evaluate different diagnostic tests and did not involve live animals. The research protocol was approved by the University of Missouri Institutional Biosafety Committee (protocol number: 11721 2.2).

Comparison of two different immunoassays

For TeSeE ELISA, 200 ± 20 mg of RPLN tissue was trimmed using a disposable scalpel and transferred to a 1.5 mL tube containing grinding beads (Bio-Rad Laboratories). The sample was homogenized according to the manufacturer's instruction (Bio-Rad Laboratories). Approximately 250 μL of the homogenate was incubated with an equal volume of Reagent A containing Proteinase K at 37°C for 10 min followed by adding 250 μL of Reagent B to the tube and centrifuging for 7 min at 15,000 g. The precipitated protein was mixed with 25 μL of Reagent C, incubated for 5 min at 100°C , and mixed again via a brief vortex agitation. The purified sample was diluted with 125 μL of Reagent R6. ELISA procedures were performed as follows: transferring 100 μL of the diluted sample to a designated well of the TeSeE ELISA plate (Bio-Rad Laboratories), incubating for 30 min at 37°C followed by three washes with Reagent R2, adding 100 μL of conjugate (Reagent R7) to each well, incubation for 30 min at 4°C followed by five washes with Reagent R2, adding 100 μL of substrate solution, incubating in dark at 20°C for 30 min, adding 100 μL of stop solution, and determining the optical density using a microplate reader (iMark, Bio-Rad Laboratories) equipped with the TeSeE detection software at 450 nm with a reference wavelength of 620 nm.

For CWD Ag-ELISA, 250 ± 50 mg of RPLN was trimmed using a disposable scalpel and transferred to a 1.5 mm ceramic bead tube containing 900 μL of ddH₂O. The sample was homogenized using a Bead Mill homogenizer (VWR Life Science) for two cycles of 1 min at a speed of 6.5 m/s with a 10-second dwell halfway through the 1-minute run. ELISA procedures were carried out as follows: mixing 100 μL RPLN homogenate with 25 μL of

sample diluent, transferring 100 μL of the mixture to a designated well of the ELISA plate, incubating at 25°C for 1 h, washing the plate six times with 350 μL of 1 x wash solution, adding 100 μL of conditioning buffer to each well followed by incubating at 20°C for 10 min and three washes with 1 x wash buffer 2, adding 100 μL of HRPO-conjugated anti-PrP^{Sc} antibody to each well followed by incubating at 20°C for 1 h and five washes with 350 μL wash solution 2, adding 100 μL of TMB substrate to each well followed by incubating at 20°C for 15 min, adding 100 μL of 1 N HCl stop solution, and determining optical density using a microplate reader (ELX800, BioTek Instruments) at 450 nm with a reference wavelength of 650. In addition, serially diluted CWD+RPLN samples and undiluted CWD- RPLN samples were tested in duplicate using the HerdChek CWD Ag-ELISA kit which served as a basis to evaluate MEMS biosensor sensitivity.

Triplicate homogenates of each RPLN were processed and subjected to ELISA analysis along with appropriate positive and negative controls. Qualitative results (positive or negative) were interpreted by following the corresponding manufacturers' specific instructions. For semi-quantitative analysis, sample/positive control (S/P) ratio for each RPLN sample was calculated using the following formula: $(\text{Sample OD} - \text{Negative Control OD}) / (\text{Positive Control OD} - \text{Negative Control OD})$. Intra-assay coefficient of variation (CV) for each ELISA method was determined as the average CV for the 30 CWD+RPLN samples and individual CV was calculated using the formula $\text{CV} = \delta / \mu$ where δ = standard deviation and μ = mean S/P value of the triplicate tests for each RPLN.

MEMS biosensor design, fabrication, and analysis

The microelectromechanical system (MEMS)-based biosensor was designed with the functions of measuring impedance changes induced by the binding of antigen to antibody coated on the detection electrodes [35]. The fabrication of the sensors was described previously [35]. In brief, the biosensor consisted of a focusing region with a sample inlet and waste outlet, a trapping region, and a detection region with an antibody inlet, an antibody outlet, and a sample outlet. The focusing region was responsible for concentrating prions in the microfluidic channel using two arrays of focusing electrodes wired in a parallel configuration. The unique features of this region included the distinct thick ramp-down electrode pairs along with the tilted thin film fingers that had varying lengths and widths allowing the centration of prions to the centerline of the microchannel while discarding over 90% of fluid in the original sample. The trapping region consisted of gold electroplated and non-parallel electrode pairs surrounding the detection electrodes to maximize prion

concentration in the detection region. The trapping electrodes use positive dielectrophoresis (p-DEP) forces to catch prions on the surface of the interdigitated electrode arrays (IDE). The detection region contained a fluidic microchannel with 2 sets of IDE arrays with one set being

coated with anti-prion antibody and the other set serving as a negative control. The design of the MEMS-based biosensor is illustrated in Fig. 1.

The MEMS biosensors were used to test serially diluted CWD+RPLN samples (10^{-0} to 10^{-3}) and undiluted

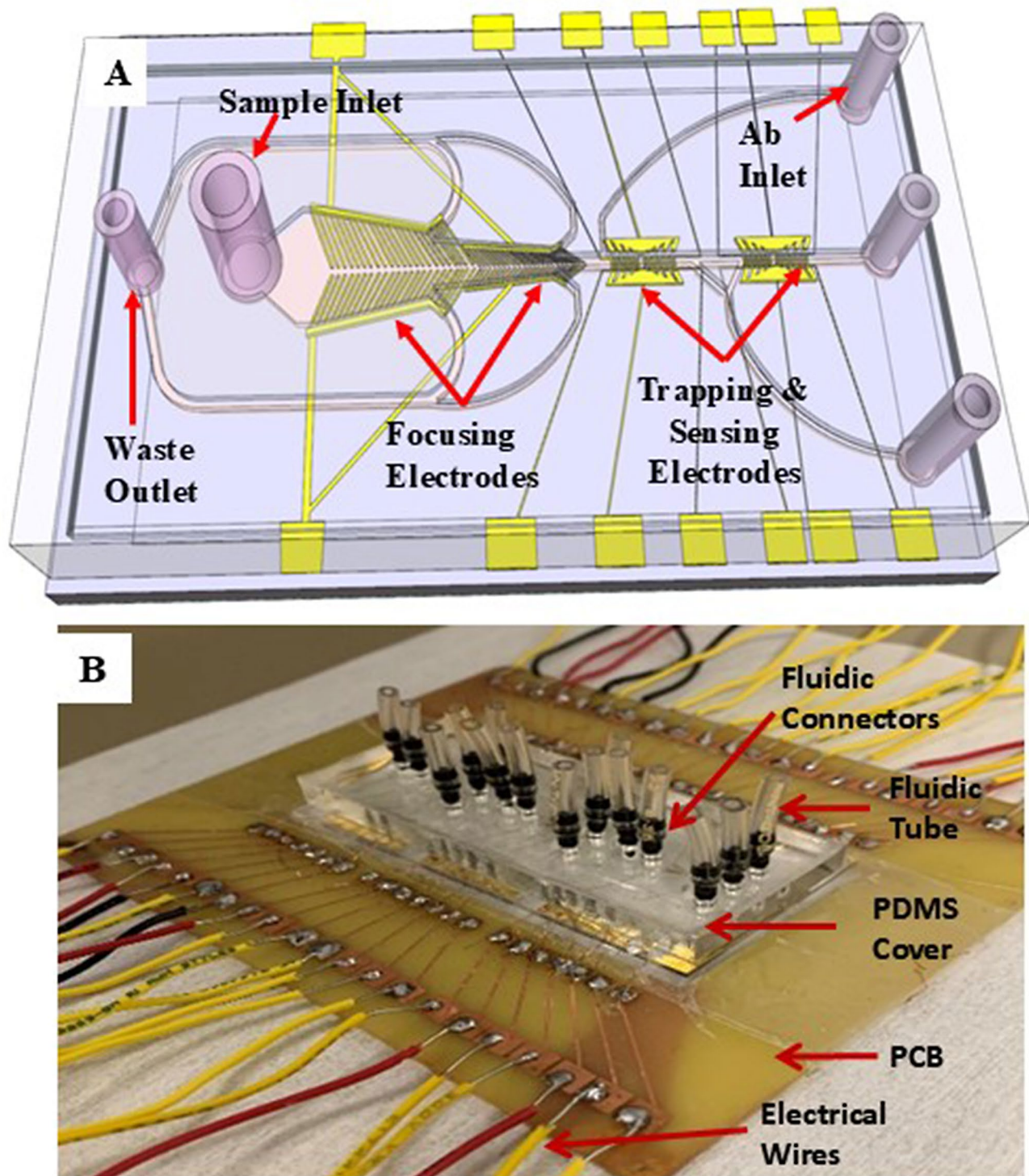


Fig. 1 Description of the MEMS biosensor. A: 3-D review of the sensor. B: A photograph of the packaged biosensor on a printed circuit board (PCB)

CWD- RPLN samples. In brief, anti-prion monoclonal antibody (mAb) (F99/97.6.1, VMRD) was conjugated to a crosslinker, namely sulfo-succinimidyl 6-[3-(2-pyridyl-dithio) propionamido] hexanoate (sulfo-LC-SPDP) as described previously [35]. The conjugated mAb (100 μ L, 2 μ g/mL) was loaded into the antibody inlet of the biosensor and incubated at room temperature for 60 min to allow attaching to the detection electrodes. After sucking the fluid containing unbound mAb from the antibody outlet, the impedance was measured for both detection and control electrodes. Then RPLN homogenate (100 μ L) was injected into the sample inlet and allowed to enter the focusing region while waste fluid was sucked out through the waste outlet. The concentrated sample was then pushed to the trapping and detection regions using a function generator (Keysight Technologies) with optimized alternating current (AC) voltages at specific frequencies (5 V_{p-p} at 6 MHz). The biosensor was kept at room temperature for 30 min for prion-mAb binding. After washing the detection channel with dH₂O to remove the unbound prions, the impedance was measured again for both detection and control electrodes at frequencies (the rate at which alternative AC current changes direction per second) from 100 Hz to 100,000 Hz using an Impedance Analyzer (Keysight Technologies). The difference between impedance induced by antibody alone and by antibody-antigen interaction was calculated and used to determine the positive or negative status of a sample. The cutoff for a positive response was set as the impedance of all negative samples + 5 SD.

RT-QuIC analysis

RPLN homogenates were serially diluted in PBS from 10^{-2} to 10^{-5} and analyzed by RT-QuIC. RT-QuIC master mix contained 20 mM Na₂HPO₄, 320 mM NaI, 1 mM EDTA, 50 μ M Thioflavin T (Sigma), and 0.1 mg/ml RT-QuIC rPrP substrate (Syrian Hamster rPrP: 90–231 developed by CWD Evolution and distributed by VMRD, Pullman, WA, USA). RT-QuIC reaction consisting of 95 μ L master mix and 3 μ L RPLN homogenate was carried out in a 96-well black/clear bottom plate (Greiner Bio-One) that was incubated at 42°C in a FLUOstar Omega microplate reader (BMG Labtech). RT-QuIC program was set at 1 min rest followed by 1 min shaking at 700 rpm with fluorescence readings every 15 min (450/460 nm excitation, 480 nm emission, bottom read, 20 flashes per well, manual gain 1,800) for 72 h. Data was analyzed using the MARS analytical software (BMG Labtech). Threshold for a positive reaction was established as 2 x mean value of the first 10 fluorescence readings of each well. At a specific dilution, a sample was defined as RT-QuIC+ if two out of three reactions were positive. Rate of amyloid formation (RAF) was calculated as the inverse of time (1/h) to threshold (ToT). For the

ease of data analysis, 0.001 was assigned as the RAF for a negative reaction. Each run included triplicate sample wells and duplicate positive (ELISA+/IHC+RPLN homogenate) and negative control (ELISA-/IHC- RPLN homogenate) wells.

Sensitivity and specificity

Diagnostic sensitivity was defined as the percentage of CWD+RPLN samples that were correctly identified by RT-QuIC or MEMS biosensor. Diagnostic specificity was defined as the percentage of CWD- RPLN samples that were correctly excluded by RT-QuIC or MEMS biosensor.

Statistical analysis

To evaluate the sensitivity of MEMS biosensor against CWD Ag-ELISA, we compared the qualitative results (positive or negative) for CWD+RPLN samples at the following dilutions: 10^{-0} , 10^{-1} , 10^{-2} , and 10^{-3} [36, 37]. We examined CWD positivity of the 30 CWD+RPLN samples for a total of 480 data points (30 samples x 2 repeats x 4 dilutions x 2 methods). Because ELISA results were positive for all CWD+RPLN samples at 10^{-0} dilution and negative at 10^{-3} dilution, we only estimated ELISA sensitivity at 10^{-1} (51 positive) and 10^{-2} (16 positive) dilutions using a generalized linear mixed model with a Bernoulli distribution in a Bayesian framework. Response data were sample positivity results, y_{ij} . Test type, either BioSensor or ELISA, was included as a fixed intercept, α_i , and deer identity was included as a random effect centered on zero, β_j .

$$y_{ij} \sim \text{Bernoulli}(p_{ij})$$

$$\text{logit}(p_{ij}) = \alpha_i [\text{Dilution}_{ij}] + \beta_j [\text{Deer}_{ij}]$$

$$\alpha_i \sim \text{normal}(0, 0.001)$$

$$\beta_j \sim \text{normal}(0, \sigma)$$

$$\sigma \sim \text{uniform}(0, 10)$$

Models evaluating sensitivity and S/P ratio results for CWD testing methods all converged based on Rhat values of ≤ 1.1 for estimated parameters and other diagnostics. Models fit to the data was also reasonable ($BPV_{\text{sensitivity}} = 0.374$, $BPV_{\text{sp ratio}} = 0.651$)

Results

CWD ELISA and Ag-ELISA

Upon analysis using TeSeE ELISA and CWD Ag-ELISA, all 30 CWD+RPLN samples were positive and 30 CWD-RPLN samples were negative by both methods based on

the criteria set by the manufacturers of these kits. Sample over positive control (S/P) values were also examined (Fig. 1). For the 30 CWD+RPLN samples, TeSeE ELISA resulted in greater SP ratios ranging from 0.56 to 3.13 for individual assays with an average SP of all assays for all samples being 2.68 ± 0.64 , compared to CWD Ag-ELISA from 0.71 to 1.79 for individual assays with an average SP of 1.47 ± 0.25 . For the 30 CWD- RPLN samples, S/P values resulted from both methods were near zero (TeSeE ELISA: 0.003 ± 0.004 and CWD Ag-ELISA: 0.003 ± 0.014). Intra-assay variations for each RPLN sample were observed, especially with TeSeE ELISA (Fig. 2). The coefficient of variation (CV) in S/P ratios for the TeSeE ELISA was 16.52% whereas CV for and CWD Ag-ELISA was 9.49%. However, the variations did not affect the qualitative results (CWD positive or negative) as determined using the corresponding manufacturer's cutoff for positive.

MEMS biosensor performance

The impedance ($M\Omega$) changes induced by representative CWD+and CWD- RPLN samples at various

frequencies are shown in Fig. 3. At frequencies from 100 to 10,000 Hz, concentration-dependent impedance changes were detected for the two CWD+RPLN samples. The differences between 10^{-0} dilution and 10^{-3} dilution were 12- to 17-fold for a strong CWD+sample (Fig. 3A) and 14- to 24-fold for a weak CWD+sample at frequencies (Fig. 3B) from 100 to 10,000 Hz. Meanwhile, a minimum of 11-fold difference between 10^{-3} dilution and the control electrodes was detected (Fig. 3A and B) at frequencies from 100 to 10,000 Hz. At 100,000 Hz, impedance change diminished to a baseline level and no difference was detected between sample dilutions (Fig. 3A, B). In contrast to the CWD+RPLN samples, the two CWD- RPLN samples without dilution induced baseline impedance changes at frequencies from 100 to 100,000 Hz (Fig. 3C).

Next, the impedance changes triggered by the 30 CWD+RPLN samples at dilutions from 10^{-0} to 10^{-3} and the CWD- samples without dilution were measured at a frequency of 1000 Hz (Fig. 4). The average impedance for the 30 CWD+samples was $11.15 \pm 0.98 M\Omega$ at 10^{-0} dilution, $7.70 \pm 0.65 M\Omega$ at 10^{-1} dilution, $2.74 \pm 1.45 M\Omega$

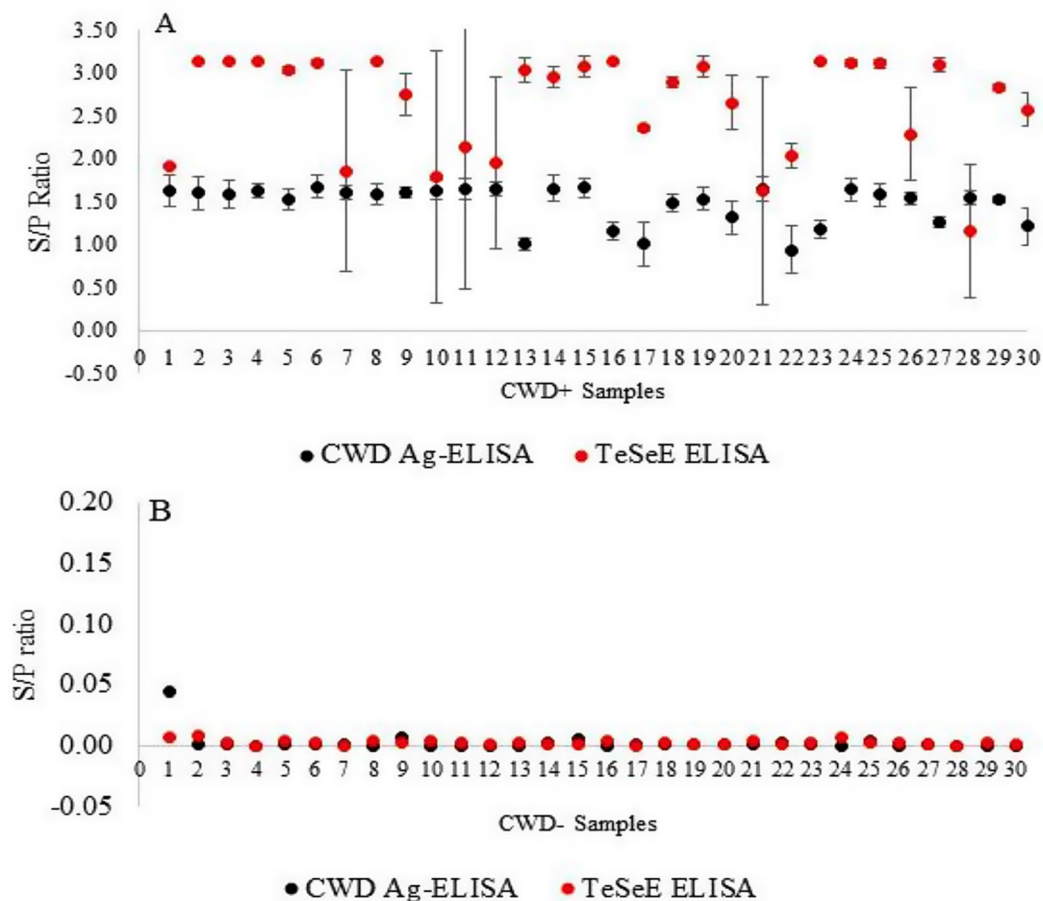


Fig. 2 S/P ratios generated by using TeSeE ELISA and CWD Ag-ELISA. (A): S/P ratios of 30 CWD+ RPLN samples. (B): S/P ratios of 30 CWD- RPLN samples. Data shown are mean values of triplicate tests $\pm$ SD

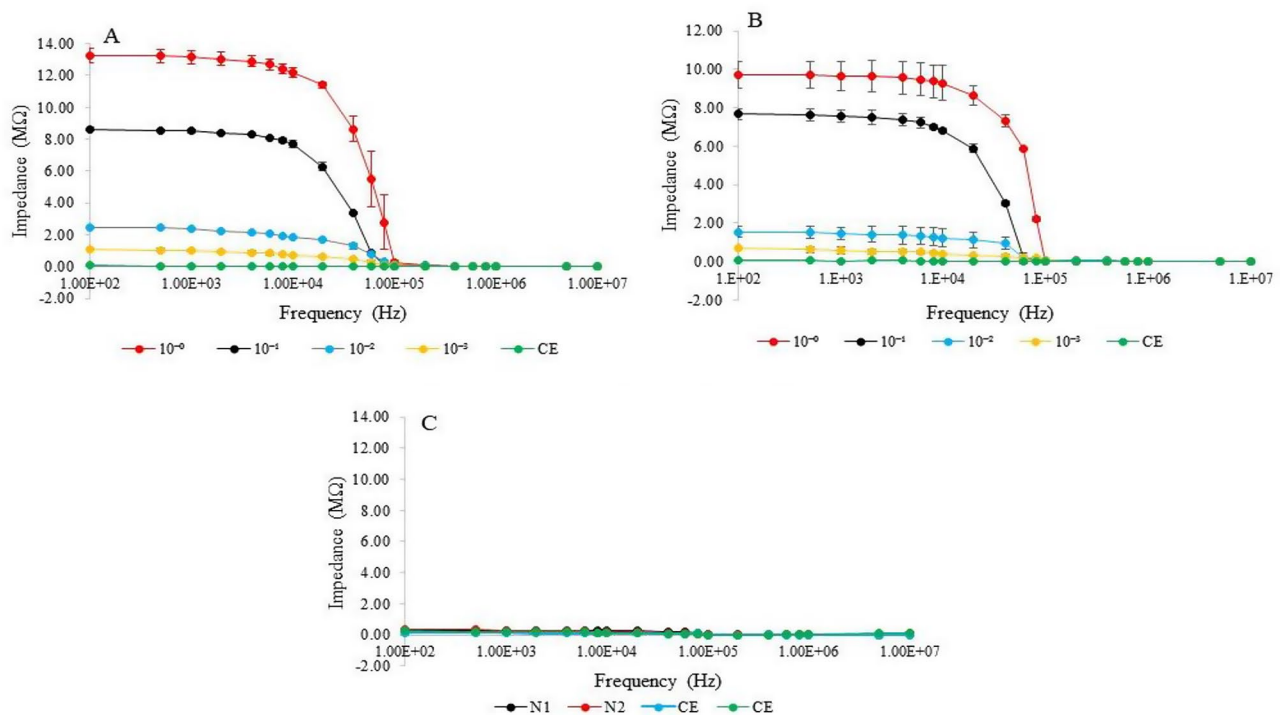


Fig. 3 Impedance change induced by representative RPLN samples at various frequencies (10^2 to 10^7 Hz). **(A):** Strong CWD+ by IHC. **(B):** Weak CWD+ by IHC. **(C):** two CWD- samples. CE: control electrodes. CWD+ samples at dilutions from 10^{-0} to 10^{-3} and CWD- samples with no dilution were analyzed by the MEMS biosensor. Data shown are average impedance from two independent runs

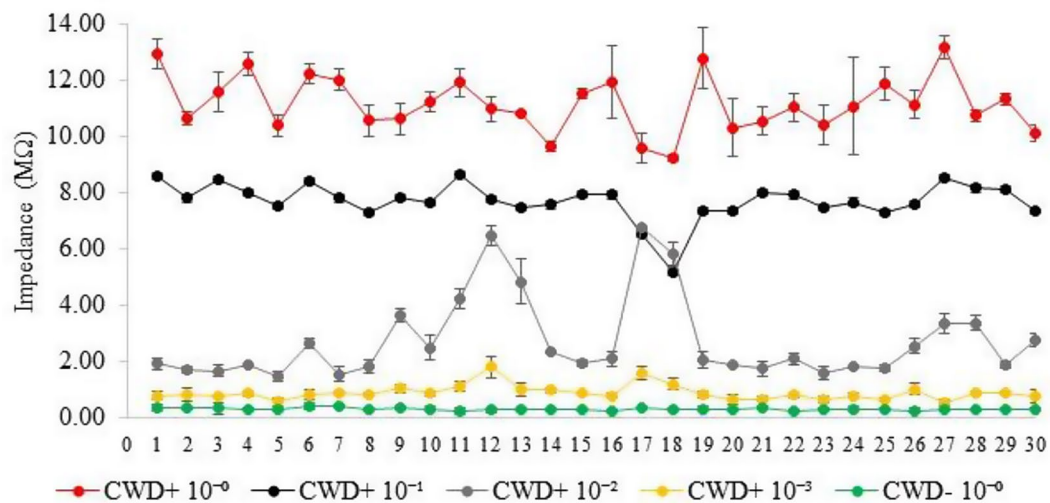


Fig. 4 Impedance change of 30 CWD+ and 30 CWD- RPLN samples. CWD+ samples at dilutions from 10^{-0} to 10^{-3} and CWD- samples without dilution were analyzed by the MEMS biosensor at a frequency of 1000 Hz. Data shown are average impedance from two independent runs

at 10^{-2} dilution, and 0.90 ± 0.26 MΩ at 10^{-3} dilution. In contrast, the average impedance of the 30 undiluted CWD- samples was 0.32 ± 0.04 MΩ, indicating that the MEMS biosensor analysis was able to distinguish positive and negative samples even when the positive samples were diluted 1,000 times.

The sensitivity of MEMS biosensor in comparison to CWD Ag-ELISA was summarized in Table 1. Statistical

analysis indicated that the true positive rate was 100% for MEMS biosensor on CWD+RPLN samples at dilutions from 10^{-0} to 10^{-3} , 100% for CWD Ag-ELISA on samples with no dilution, and 0% for CWD Ag-ELISA for samples at 10^{-3} dilution. The estimated positivity rate for CWD Ag-ELISA was 0.849 (95% CRI: 0.134, 1.000) at 10^{-1} dilution and 0.266 (95% CRI: 0.000, 0.893) at 10^{-2} dilution.

Table 1 Positive detection rate of MEMS biosensor and CWD Ag-ELISA *

Test	Dilution	# positives	Mean	0.025	0.975
MEMS biosensor	10 ⁻⁰	60	1.000	1.000	1.000
MEMS biosensor	10 ⁻¹	60	1.000	1.000	1.000
MEMS biosensor	10 ⁻²	60	1.000	1.000	1.000
MEMS biosensor	10 ⁻³	60	1.000	1.000	1.000
CWD Ag-ELISA	10 ⁻⁰	60	1.000	1.000	1.000
CWD Ag-ELISA	10 ⁻¹	51	0.849	0.134	1.000
CWD Ag-ELISA	10 ⁻²	16	0.266	0.000	0.893
CWD Ag-ELISA	10 ⁻³	0	0.000	0.000	0.000

* Thirty CWD+ (ELISA+/IHC+) RPLN samples were subjected to 10-fold serial dilutions from 10⁻⁰ to 10⁻³ and tested in duplicate by MEMS biosensor and CWD Ag-ELISA. The number of positive results under each testing condition and the estimated mean and bounds of the 95% credible intervals (CRI; 0.025, 0.975) for sensitivity are provided

RT-QuIC performance

The percentage of RT-QuIC positive reactions and the average time to threshold (ToT) for all CWD+ and CWD- RPLN samples were summarized in Table 2. At 10⁻² sample dilution, 77.78% of RT-QuIC reactions for CWD+ RPLN samples were positive while 20% of reactions for CWD- RPLN samples were also positive. Wide variations in ToT were observed among both CWD+ and CWD- RPLN samples. When sample dilutions increased to 10⁻⁴ and 10⁻⁵, 100% of the reactions for CWD+ RPLN samples were positive and 8.89% and 2.22%, respectively, of the reactions for CWD- RPLN samples were positive. With the CWD- RPLN samples showing false positivity, only one of the triplicate reactions per sample turned positive. At 10⁻⁴ dilution, the average ToT for all CWD+ RPLN samples was 24.55 ± 2.52 h while the average ToT for CWD- samples was 50.99 ± 15.05 h with one of the eight positive reactions having a ToT of 25.5 h. At 10⁻⁵ dilution, clear differences in ToT were detected between CWD+ RPLN samples (22.52 ± 2.76 h) and CWD- RPLN samples (49.05 ± 6.72 h).

The seeding activities of all samples, expressed as the rate of amyloid formation (RAF, 1/ToT), are presented

in Fig. 5. At 10⁻² dilution, wide inter- and intra-sample variations were observed for samples derived from both CWD+ and CWD- deer. As sample dilution increased to 10⁻⁵, RAF values became more uniform among samples and individual reactions for the same sample.

Diagnostic sensitivity and specificity of RT-QuIC and MEMS biosensor

With a cutoff of the mean impedance of all negative samples + 5 SD (0.52), the MEMS biosensor demonstrated a sensitivity of 100% on RPLN sample at dilutions from 10⁻⁰ to 10⁻³ and a specificity of 100% on undiluted RPLN samples (Fig. 6A). Using a threshold of 2 x mean value of the first 10 readings and a final cutoff of 2/3 positive reactions, the diagnostic sensitivity of RT-QuIC was 93.33% at 10⁻² dilution, 100% at 10⁻³ dilution, 100% at 10⁻⁴ dilution, and 100% at 10⁻⁵ dilution. The specificity ranged from 83.33% at 10⁻² dilution, 93.33% at 10⁻³ dilution, 96.67% at 10⁻⁴ dilution, and 100% at 10⁻⁵ dilution: indicating that 10⁻⁵ was the ideal dilution for RPLN RT-QuIC under the experimental conditions described in this study (Fig. 6B).

Discussion

The diagnosis of cervid CWD relies heavily on IHC and ELISA. These methodologies are suitable for postmortem detection of CWD prion in the brain and RPLN of the affected cervids but may not work well on samples with a low prion load. New technologies, such as RT-QuIC and MEMS biosensor have recently been developed to detect CWD prion. In this study, we compared the performance of two different immunoassays, RT-QuIC and a MEMS biosensor for the detection of CWD prion in RPLN samples.

With the two immunoassays, TeSeE ELISA, designed for diagnosing bovine spongiform encephalopathy (BSE) and scrapie, is approved by the USDA for the detection of CWD in farmed cervids (Bio-Rad Laboratories User Manual). This method includes the following procedures:

Table 2 RT-QuIC reactivity of RPLN samples at different dilutions*

Dilution	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵
CWD+ RPLN Samples				
# of Pos	70	88	90	90
% of Pos	77.78	97.78	100.00	100.00
% of False Neg	22.22	2.22	0.00	0.00
ToT (range)	21.98 ± 15.29 (9.75 to 68.00)	21.02 ± 2.94 (11.25 to 28.75)	24.55 ± 2.52 (17.00 to 28.25)	22.52 ± 2.76 (16.75 to 29.25)
CWD- RPLN Samples				
# of Neg	72	79	82	88
% of Neg		80.00	87.78	91.11
% of False Pos	20.00	12.22	8.89	2.22
ToT (range)	56.42 ± 10.27 (34.75 to 70.25)	51.93 ± 15.06 (23.75 to 70.00)	50.59 ± 15.05 (25.50 to 68.25)	49.05 ± 6.72 (44.75–54.25)

*Thirty CWD+ (ELISA+/IHC+) and 30 CWD- (ELISA-) RPLN samples were subjected to 10-fold serial dilutions. Diluted samples (10⁻² to 10⁻⁵) were tested in triplicates by RT-QuIC. The total number of RT-QuIC reactions for CWD+ or CWD- samples at each dilution was 90 (30 samples x 3 wells). Percentage of positive or negative reactions at each dilution was calculated as # of positive or negative / 90 x 100

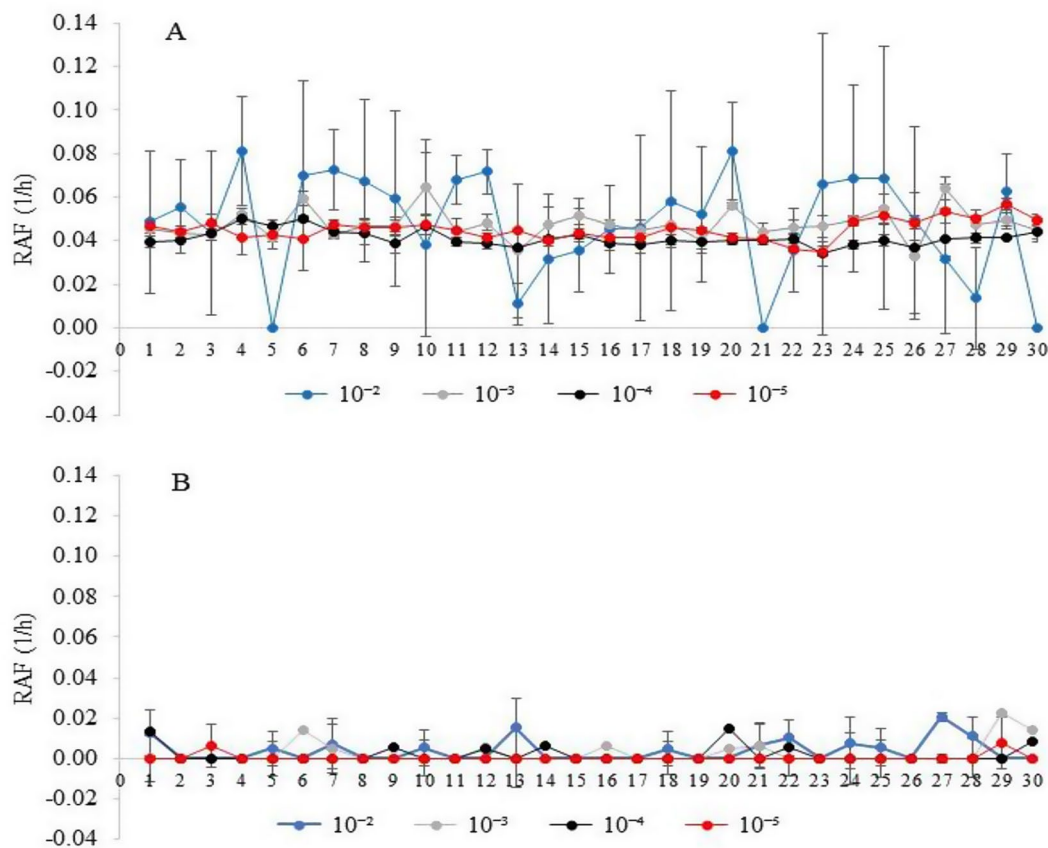


Fig. 5 Rate of amyloid formation. A: 30 CWD+ RPLN samples. B: 30 CWD- RPLN samples. Samples at dilutions from 10^{-2} to 10^{-5} were analyzed by RT-QuIC. Data shown are mean rate of amyloid formation (RAF) $\pm$ SD and 0.001 was assigned to a negative reaction for ease of analysis

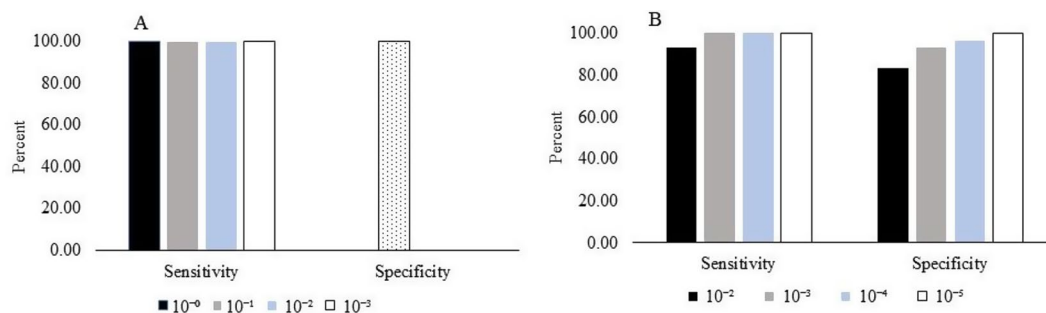


Fig. 6 Diagnostic sensitivity and specificity of MEMS biosensor and RT-QuIC. (A): MEMS biosensor analysis of CWD+ samples at dilutions from 10^{-2} to 10^{-5} and CWD- samples with no dilution. (B): RT-QuIC analysis of CWD+ and CWD- samples at dilutions from 10^{-2} to 10^{-5} . Sensitivity was defined as the percentage of CWD+ RPLN samples ($n=30$) that were correctly detected by MEMS biosensor or RT-QuIC. Specificity was defined as the percentage of CWD- RPLN samples ($n=30$) that were correctly excluded by MEMS biosensor (only for undiluted samples) or RT-QuIC

tissue dissection, homogenization, purification of Prp^{Sc} in tissue homogenate, and subsequent detection of prion by ELISA. The CWD Ag-ELISA is an antigen-capture enzyme immunoassay for the detection of Prp^{Sc} in white-tailed deer and mule deer as well as elk in the late stage of the disease (IDEXX CWD Ag-ELISA User Manual). CWD Ag-ELISA does not involve protein purification which renders the test to be operationally efficient, a characteristic favored by laboratories that

analyze thousands of samples per day. Currently, the CWD Ag-ELISA kit is not approved by USDA for the diagnosis of CWD in farmed cervids, but some states including Missouri allow the use of this kit to screen wild deer populations. To compare the performance of these immunoassays, we tested archived RPLN samples with known CWD status. Our results demonstrated that both TeSeE ELISA and CWD Ag-ELISA kits correctly detected all positive and negative RPLN samples

and the subsamples. We also examined the S/P ratios and found that TeSeE kit yielded higher S/P values along with greater intra-assay coefficient of variation, compared to CWD Ag-ELISA kit. Because both immunoassays worked equally well for qualitatively detecting CWD prion in RPLN samples and CWD Ag-ELISA was easy to perform, we relied on CWD Ag-ELISA for subsequent evaluation of MEMS biosensor.

With MEMS biosensor, the optimal conditions, including antibody concentration, cross-linking, and incubation time, have been extensively studied [33–35]. Previously, we evaluated the MEMS biosensor using the positive control antigen from the CWD Ag-ELISA kit and a limited number of RPLN samples [35]. In this study, we validated the MEMS biosensor using 30 CWD+ and 30 CWD-RPLN samples and assessed its performance based on the initial results generated using ELISA in combination with IHC. Our results indicated that the biosensor correctly identified all CWD+ and CWD- samples. Because both MEMS biosensor and immunoassays are based on antibody-antigen binding, we further assessed the sensitivity of the biosensor relative to CWD Ag-ELISA. The 100% detection rate for MEMS biosensor versus 0% detection rate for CWD Ag-ELISA in CWD+RPLN samples at 10^{-3} dilution confirmed our previous finding that the MEMS biosensor is highly sensitive [35]. It must be noted that ELISA assays were designed to test brain and RPLN samples with no dilution and the comparative study was simply aimed at evaluating the potential suitability of the biosensor for detecting low level PrP^{Sc} in RPLN samples. In this study, we did not evaluate MEMS biosensor using serially diluted CWD- RPLN samples because undiluted CWD- RPLN samples induced only baseline impedance changes. Unlike RT-QuIC, MEMS biosensor does not convert PrP^C to PrP^{Sc} which reasonably preclude the possibility that diluting negative samples would reverse the impedance response triggered by Ab-Ag binding.

With RT-QuIC analysis, we used NaI as the main ionic component in assay buffer as it was shown by our previous work that NaI significantly improved RT-QuIC sensitivity [38]. We used a stringent threshold for reaction positivity (2 x mean value of the first 10 fluorescence readings of each well) to minimize the effect of sample matrix on prion RT-QuIC reaction. We also evaluated effect of sample dilution on RT-QuIC sensitivity and specificity. Our data indicated that the rate of false negative reactions decreased from 21.98% at 10^{-2} dilution to 0% at 10^{-4} and 10^{-5} dilutions and the rate of false positive reaction reduced from 56.42% at 10^{-2} dilution to 8.89% at 10^{-4} dilution and 2.22% at 10^{-5} dilution. With the false positive reactions seen with CWD- RPLN samples at high dilutions, it was necessary to set specific interpretive criteria for RPLN RT-QuIC. Thus, we examined both ToT, also known Ct or ThT, and the ratio of positive

to total reactions for each sample. Because one reaction for a CWD- sample had a short ToT (25.5 h) which fell within the ToT range for CWD+RPLN samples at 10^{-4} dilution, we did not use ToT as an interpretive criterion. On the other hand, false positivity was detected in only 1/3 reactions at both 10^{-4} and 10^{-5} dilutions, we selected positivity in 2/3 of reactions as a criterion for a sample to be called as positive. Based on above parameters, RT-QuIC reached 100% sensitivity and specificity on RPLN samples at 10^{-4} and 10^{-5} dilutions. Although a number of publications have demonstrated that RT-QuIC is suitable for detecting CWD prion in RPLN and other samples [29, 31, 32], assays interpretive criteria have not been established. Our previous data from RT-QuIC analysis of deer platelet samples showed that 10^{-2} dilution of the samples produced most reliable results. The findings of this study indicate that testing RPLN at 10^{-4} or 10^{-5} dilution with triplicate wells can achieve a satisfactory diagnostic outcome. Given that testing each sample at multiple dilutions with greater numbers of replicates is prohibitively expensive for routine diagnostic purposes, we recommend testing RPLN at 10^{-4} or 10^{-5} with three reactions.

Conclusion

The sensitivity and specificity as well as the performance characteristics of two immunoassays, MEMS biosensor, and RT-QuIC were evaluated by testing 30 CWD+ and 30 CWD- RPLN samples from white-tailed deer. TeSeE ELISA yielded greater S/P values along greater intra-assay CV, compared to CWD Ag-ELISA. The MEMS biosensor achieved 100% sensitivity with CWD+RPLN samples at 10^{-0} to 10^{-3} dilutions and 100% specificity with CWD-RPLN samples without dilution. RT-QuIC also reached a 100% sensitivity and 100% specificity at a sample dilution level of 10^{-4} or 10^{-5} , using the following criteria (1) time to threshold (ToT) for a positive reaction being 2 x the mean value of the first 10 fluorescence readings of a well and (2) cutoff for a sample to be called positive being that 2/3 reactions are positive. Data from this study indicate that MEMS biosensor is a reliable portable tool for CWD diagnosis and RT-QuIC can be used for routine testing of CWD if appropriate testing parameters and interpretive criteria are set.

Abbreviations

CWD	Chronic wasting disease
CV	Coefficient of variation
ELISA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
Hz	Hertz
IDE	Interdigitated electrode array
IHC	Immunohistochemistry
mAb	monoclonal antibody
NaCl	Sodium chloride
NaI	Sodium iodide
PBS	Phosphor buffered saline
PMCA	Protein misfolding cyclic amplification

PrP ^C	Prion protein-cellular
PrP ^{Sc}	Prion protein-scrapie
rPrP	Recombinant prion protein
RAF	Rate of amyloid formation
RPLN	Retropharyngeal lymph node
RT-QuIC	Real-time quaking induced conversion
ToT	Time to threshold
Vp-p	Peak to peak voltage
WB	Western blotting

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Author contributions

EK processed RPLN samples for RT-QuIC and MEMS Biosensor analysis and conducted RT-QuIC analysis. S.A.M. and A.A. fabricated the biosensors and performed biosensor testing and analysis of RPLN samples. K.A. performed immunoassays. E.A.S. performed statistical analysis. A.M. designed MEMS biosensor and oversaw biosensor fabrication, testing, and analysis. S.R., M.Z.Z. and S.Z. conceived the project. S.Z. coordinated the work by different team members. M.Z.Z. and S.Z. drafted the manuscript.

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Data availability

Data are provided within the manuscript and supplementary information files.

Declarations

Ethics approval and consent to participate

This research did not involve live animals. Retropharyngeal lymph nodes were collected from hunter-harvested dead deer by MDC as part of Misouri CWD surveillance program. Sample collection and submission protocols were reviewed and approved by MDC Science Committee. Comparative study protocol was approved by the University of Missouri Institutional Biosafety Committee (protocol number: 11721.2.2).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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