

Chronic Wasting Disease Options of Surveillance in Carpathian Cervids: from the Identification of Characteristic Microscopic Pathologic Changes to Prionic Seeded Conversion and Amplification Assays

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Abstract. *This paper describes the current diagnostic methods of Chronic Wasting Disease (CWD) in cervides used between 2013 and 2017 in Romania. The active surveillance of CWD involves the targeted groups screening by using rapid diagnostic tests (e.g., antigen capture enzyme immunoassay). If the first test does not provide certain negative results, then the confirmatory methods have been used, i.e. histopathology, immunohistochemistry and Western immunoblotting. These tests did not lead to the detection of CWD prions (PrP^{CWD}) in Romania. This may be due to the absence or insufficient quantity of PrP^{CWD} in samples, below the threshold of confirmatory tests.*

Keywords: CWD, transmissible spongiform encephalopathy, surveillance, diagnostic

1. Introduction

The Chronic Wasting Disease (CWD) is a transmissible spongiform encephalopathy (TSE) of the cervids (deer, elk, moose, and reindeer), characterised by spongy degeneration of the brain with progressive emaciation, abnormal behaviour and death as a consequence of the progressive loss of bodily functions [1, 2]. The first description of CWD was recorded in North American deer (*Odocoileus hemionus hemionus*) hosted by a northern Colorado research facility, in the late 1960s [3]. In the following years, the CWD emergence has been recorded in several North American, Asian and European locations as a consequence of the increased surveillance and the spread of the disease by natural migration or translocation of the infected cervids, induced by humans [4]. In time, several risk factors of CWD spreading have been identified, such as failure to separate farmed and free-ranging cervids, high deer density, genetics, species-specific social organisation, sex-related behaviours, natural or man-mediated animal aggregation, consumption of forage grown on contaminated soil, fallen stock or inappropriate disposal of carcasses and slaughter by-products [4, 5].

The infectious agent of CWD (PrP^{CWD}) is a misfolded form of the endogenous cellular prion protein PrP^C [6]. Prusiner (1982) defined prions as “proteinaceous infectious particles” that are devoid of nucleic acids [7]. The propagation of pathologic isoforms involves the PrP^C coercion to adopt the conformation of the infectious isoform, respectively to reproduce their secondary, tertiary and quaternary conformational structures [8,9]. PrP^C is a glycosylphosphatidylinositol-anchored membrane protein, a class of proteins containing a soluble protein attached by a conserved post-translational glycolipid anchor to the outer leaflet of the plasma membrane of neurons, glial cells, and other cell types [10].

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In the last twenty years, the PrP^{CWD} detection methods have progressed significantly, from the identification of the characteristic microscopic pathologic changes to antibody-antigen dependent immunoassays (e.g., EIA - enzyme immunoassay, IHC - immunohistochemistry, WB - western blotting), and, more recently, researchers have begun incorporating methods of the seeded amplification (e.g., PMCA - protein misfolding cyclic amplification, RT-QuIC - real time quaking-induced conversion) [11]. The most commonly EIAs are HerdChek BSE-Scrapie Ag Test (IDEXX) and TeSeE SAP purification-detection test (Bio-Rad). Both tests are approved as rapid detection methods of the abnormal conformer of the prion protein by using obex samples of brainstem from bovines, sheep, goats and cervids. The HerdChek BSE-Scrapie Ag Test is an antigen-capture ELISA, while the TeSeETM SAP Assay is a sandwich ELISA using two monoclonal antibodies [12, 13]. IHC and WB are highly sensitive and specific tests but are expensive, require skilled technicians, time and several working-steps, making them prone to be used more frequently in confirmatory diagnosis than in screening protocols [12, 14]. The PMCA enables exponential increase of the infectious PrP in a conceptually analogous way as DNA amplification by PCR [15]. The PMCA for CWD diagnostic use PrP^C from transgenic cervidized mice as the substrate for *in vitro* conversion of the PrP^{CWD} (serve as “seeds” for further conversion reactions) which is in a very low quantity in the test sample [11].

The RT-QuIC is generally analogous to PMCA, but employs as substrate for *in vitro* formation of the pathologic amyloid a recombinant PrP^C and the recording of the fibrils formation consists in the detection of an increase in fluorescence emission associated with the binding of thioflavin T to the amyloid fibril [16].

The active surveillance and diagnostic of CWD use biological samples from the Red deer (*Cervus elaphus* L., 1758), the European roe deer (*Capreolus capreolus* L., 1758), the Fallow deer (*Dama dama* L., 1758), the chamois (*Rupicapra rupicapra* L., 1758) and several other captive cervids. The rapid tests used for the monitoring of TSEs are those listed in Annex X of Regulation (EC) No 999/2001: “the immunoblotting test based on a Western blotting procedure for the detection of the Proteinase K-resistant fragment PrP^{Res} (Prionics-Check Western test); the sandwich immunoassay for PrP^{Res} detection (short assay protocol) carried out following denaturation and concentration steps (Bio-Rad TeSeE SAP rapid test); the microplate-based immunoassay which detects Proteinase K-resistant PrP^{Res} with monoclonal antibodies (Prionics-Check LIA test); the immunoassay using a chemical polymer for selective PrP^{Sc} capture and a monoclonal detection antibody directed against conserved regions of the PrP molecule (IDEXX HerdChek BSE Antigen Test Kit, EIA & HerdChek BSE-Scrapie Antigen); the lateral-flow immunoassay using two different monoclonal antibodies to detect Proteinase K-resistant PrP fractions (Prionics Check PrioSTRIP); the two-sided immunoassay using two different monoclonal antibodies directed against two epitopes presented in a highly unfolded state of bovine PrP^{Sc} (Roboscreen Beta Prion BSE EIA Test Kit); the sandwich immunoassay for PrP^{Res} detection with the TeSeE Sheep/Goat Detection kit carried out following denaturation and concentration steps with the TeSeE Sheep/Goat Purification kit (Bio-Rad TeSeE Sheep/Goat rapid test)” [17].

In Romania, several programs for monitoring the health of wild animals are carried out. These programs cover not only animal diseases but also the environment [18-23]. The CWD surveillance is part of the national program of TSEs surveillance, whose effectiveness was demonstrated by detecting prion infections in domestic animals [24-26].

The aim of this research is the retrospective analyses of surveillance and diagnostic methods developed for the PrP^{CWD} detection in cervids and the further progress in analyses of the infectious prions detection capabilities at the Romanian Reference Laboratory for TSEs.

2. Materials and methods

There were analysed the results of 14,983 subjects, namely Red deer, European roe deer and Fallow deer that were living in the Carpathian Mountains and the Subcarpathian regions. The following target groups of cervids were tested: sick cervids (SC), dead cervids (DC), injured or killed

cervids (IKC), hunted cervids unfit for human consumption (HUHC) and hunted cervids fit for human consumption (HFHC).

The surveillance and diagnostic methods used for the PrP^{CWD} detection by the Romanian Reference Laboratory were HerdChek BSE-Scrapie Ag Test, IDEXX, TeSeETM SAP Combi Kit, Bio-Rad, Western blotting, histopathological and histochemical analyses.

The HerdChek BSE-Scrapie Ag Test, antigen capture enzyme immunoassay EIA (IDEXX Laboratories, Westbrook, ME, USA) and TeSeE SAP purification-detection test kit, sandwich enzyme-linked immunosorbent assay (Bio-Rad Laboratories, Marnes-La-Coquette, France) were performed as per IDEXX and BioRad instructions. Both tests were used for the qualitative detection of the PrP^{CWD} in the brainstem of cervids.

The Western immunoblotting (WB) used the Bio-Rad blotting system and the brainstem of cervids as per the OIE validated and certified protocol. Briefly, the same sample homogenates prepared for Bio-Rad rapid tests were mixed with proteinase K solution (20 µL/mL) at 50 µg/mL, incubated 10 min at 37°C, precipitated with reagent B, concentrated by centrifugation 7 min at 15000 g and 20°C, clarified with the Laemmli solution, incubated 5 min at 100°C and concentrated by centrifugation 15 min at 15000 g and 20°C. Electrophoretic migration was performed at room temperature for 50 min at 150 V then immunoblotting. The protein was transferred to a polyvinylidene fluoride (PVDF) membrane at 115 V, 60 min, in transfer buffer (solution of Tris/Caps-Ethanol 15%). The membrane was then immersed in ethanol for 15 sec, rinsed in distilled water for 5 min and incubated for 30 min in blocking solution under medium agitation. The next steps consisted in the incubation of the membrane for 30 min in diluted primary antibody at room temperature under medium agitation, two washings (5 min and 10 min) with washing solution (PBS 1x + 0.1% Tween 20), under fast agitation, allowing the non-bound (or loosely bound) primary antibodies to be washed away; the incubation of the membrane for 20 min in diluted secondary antibody at room temperature under medium agitation, and other three washings (5 min, 10 min and 10 min) with the same washing solution, to allow the washing of non-bound (or loosely bound) secondary antibodies. The membrane was placed in the plastic folder, treated few seconds with ECL reagent (substrate for conjugate) and placed into the chemiluminescence reader. TSE positive samples show three typical bands (non-, mono-, and diglycoforms of the PrP^{CWD}-proteinase K digested), while the negative samples do not show any signal.

The histopathological diagnosis analysis used formalin-fixed and paraffin-embedded brainstem of the cervids, processed as per the routine, sectioned at 5 µm and stained with hematoxylin and eosin (H&E) for light microscopic examination.

Histochemical analysis was performed on the same formalin-fixed paraffine embedded brainstem used for the histopathological diagnosis, mounted on glass positive charge slides, by using an immunohistochemical staining (IHC). Slides were deparaffinised at 65°C, immersed in 100% xylene, rehydrated 2 min in 100% ethanol, 2 min in 95% ethanol and 5 min in 70% ethanol, and treated for 60 min with 88% formic acid. For specific PrP^{CWD} signal, there was used F99/97.6.1 monoclonal antibody raised against bovine residues 220–225, at the dilution 1:1250 (VMRD Inc, Pullman, Washington, USA). All tissue sections were counterstained 2 min with Mayer's hematoxylin (Dako), 5 min with 0.1% calcium bicarbonate bluing reagent and cover-slipped with mounting media.

3. Results and discussions

This study covered the surveillance and monitoring of CWD performed by the National Network for TSEs (33 county laboratories coordinated by the National Reference Laboratory for TSEs, Institute for Diagnosis and Animal Health, Bucharest, Romania) between 2013 and 2017 (table 1).

Table 1. Cervids evaluated for cwd between 2013 and 2017 in romania by using rapid tests

Year		Target Group					Total / year
		SC	DC	IKC	HUHC	HFHC	
2013	no.	3	44	18	1	2161	2227
	%	0.13	1.98	0.81	0.04	97.04	17.62
2014	no.	0	48	21	14	2395	2478
	%	0.00	1.94	0.85	0.56	96.65	19.61
2015	no.	0	35	12	1	2746	2794
	%	0.00	1.25	0.43	0.04	98.28	22.11
2016	no.	9	35	22	0	2434	2500
	%	0.36	1.40	0.88	0.00	97.36	19.78
2017	no.	0	38	24	0	2575	2637
	%	0.00	1.44	0.91	0.00	97.65	20.87
Total / group	no.	12	200	97	16	12311	12636
	%	0.09	1.58	0.77	0.13	97.43	100.00

SC – sick cervids; DC – dead cervids; IKC – injured or killed cervids; HUHC – hunted cervids unfit for human consumption; HFHC – hunted cervids fit for human consumption

The cervid groups included in this study were part of the target groups for wild and semi-domesticated cervids designed in accord with Regulation (EC) No 999/2001: (i) fallen/culled wild or semi-domesticated cervids, (ii) road- or predator-injured or killed cervids, (iii) clinical/sick wild and semi-domesticated cervids, (iv) unfit for human consumption wild hunted cervids and slaughtered semi-domesticated cervids; (v) fit for human consumption hunted wild game and slaughtered semi-domesticated cervids [26]. The CWD surveillance programs taken into consideration the targeted active surveillance of various risk groups of cervids (e.g., sick, dead or hunted cervids), with a higher interest for cervids fit for human consumption [5].

All animals were subject to the CWD active surveillance by using the rapid tests currently available, of which 41 provided initially reactive results (samples with values greater than or equal to the cut-off at the first screening test). Brain retesting was done from the same homogenate by using H&E, IHC and WB (table 2).

Table 2. Romanian cervids tested for cwd by confirmatory diagnostic methods between 2013 and 2017

Year	2013	2014	2015	2016	2017	Total
Number	7	10	7	9	8	41

In CWD the histopathological exam of the central nervous system should reveal neuronal degeneration, microcavitation of the neuropil, intracytoplasmic vacuolization, astrocytic hypertrophy and hyperplasia [11], while in IHC and WB, the protease-resistant prion protein PrP^{CWD} will be detected, which is considered a disease-specific marker [27]. In our study, for H&E (fig.1), IHC (fig.2) and WB, we were not able to detect PrP^{CWD} in samples with inconclusive results in the first screening test.

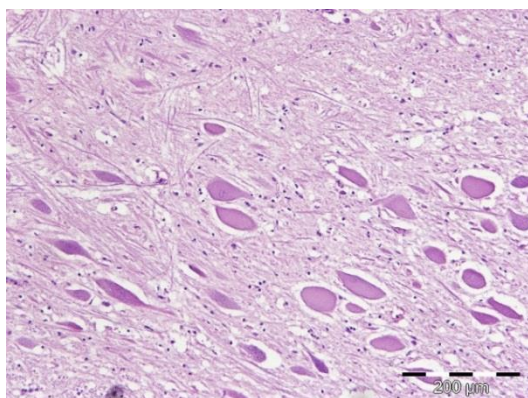


Figure 1. Histology of the obex. H&E staining without pathognomonic vacuoles in the neurons and neuropil, ×100

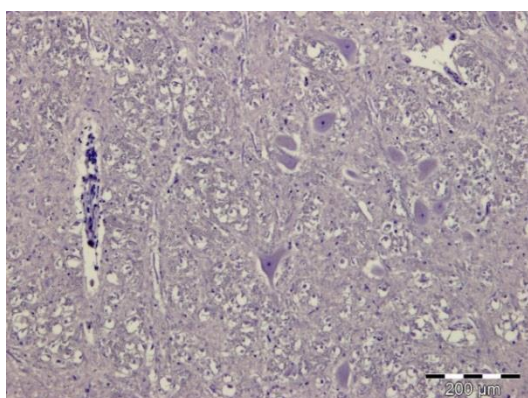


Figure 2. Immunohistochemistry of the obex. Negative results of disease-associated prion protein in brain sections at the level of the obex in cervids, IHC with F99/97.6.1 monoclonal antibody, ×100

The results could be the result of the absence of the PrP^{CWD} in all samples. However, several studies have demonstrated that the negative results could be recorded in samples with insufficient quantity of PrP^{CWD}, below the threshold of ELISA, IHC or WB. Tissues harvested from the asymptomatic infected hosts may contain low levels of PrP^{CWD} and, consequently, the efficiency of immunological tests will be diminished [28-30].

The IHC and/or WB have been considered the gold standards in the TSEs diagnostic [31], but these traditional diagnostic tests performed on nervous or lymphoid tissues seem to have a low sensitivity in the detection of CWD. Over the last decades, the sensibility of tests used in the TSE diagnostic have progressed significantly [11]. However, not all the available tests were fully standardized and evaluated in the laboratory for CWD diagnosis. The testing should be done in a way to maximise the diagnostic sensitivity and with the assumption that the extrapolation to other TSE, in a different geographical location, will provide satisfactory results [5]. Other methods with the potential to contribute to CWD detection have been developed [11], and several groups of researchers have focused in the last years in optimising variants of PMCA [32] and Real-Time Quaking-Induced Conversion (RT-QuIC) assays for detecting CWD prions [29, 33, 34]. Also, a serial-PMCA able to detect CWD prions in subclinically infected cervids has been described, and with RT-QuIC there will be possible to perform a more precise and earlier diagnosis for all neurodegenerative diseases [29, 32]. In Romania, these techniques will be added to already existing diagnostic methods, with high value in the diagnosis of bovine spongiform encephalopathy [35, 36].

The use of “new generation” cyclic amplification assays will increase the prion detection sensitivity and will improve the laboratory testing of samples with very low concentrations of PrP^{CWD}, without replacing bioassays [29].

4. Conclusions

Between 2013 and 2017, in Romania, CWD prions (PrP^{CWD}) were not detected by using high specific IHC and WB assays. This may be due to the absence or insufficient quantity of PrP^{CWD} in



samples, below the threshold of the confirmatory tests. To reduce the suspicion of negative results linked with very low concentrations of PrP^{CWD}, mainly in samples from cervids without clinical signs, new optimised methods of PrP^{CWD} detection (e.g., serial PMCA and Real-Time Quaking-Induced Conversion (RT-QuIC) assays), able to increase the prion detection sensitivity, should be included in the active surveillance and diagnosis of CWD.

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