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First results of chronic wasting disease (CWD) surveillance in the South-Eastern part of Belgium

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SUMMARY

Chronic wasting disease (CWD) has not been reported in Europe, whereas it is considered to be enzootic in free-ranging mule deer, Rocky mountain elk and white-tailed deer in the area of Colorado, Wyoming, and Nebraska, and new foci of CWD have been detected in other parts of the United States. However, no large-scale active epidemiosurveillance of European wild cervids has been installed in Europe. In accordance with the opinion of the European Scientific Steering Committee, a preliminary (active) surveillance scheme was installed, in order to improve the knowledge of the CWD status of the Belgian free-ranging cervids (roe deer and red deer). Spleen samples (n=866) of roe deer and red deer collected in the south-eastern part of Belgium, were examined for CWD using a enzyme-linked immunosorbent assay of Bio-Rad. Afterwards, the ELISA was systematically confirmed by immunohistochemistry using three antibodies, namely R524, 2G11 and 12F10. There were no indications on the occurrence of transmissible spongiform enncephalopathy (TSE) in any of the samples. A Bayesian framework was used for the estimation of the true prevalence of CWD in south-eastern part of Belgium that was estimated to have a median value of zero with a 95% percentile value of 0.00115.

Keywords: *Bayesian approach; Belgium; Brain diseases; Cervids diseases; Chronic wasting disease; CWD; Deer diseases; Prion diseases; Transmissible spongiform encephalopathy; TSE; Surveillance.*

INTRODUCTION

The animal transmissible spongiform encephalopathies (TSEs) include the archetype - scrapie in domestic sheep and goats - and animal diseases much more recently recognized, including transmissible mink encephalopathy (TME) and feline spongiform encephalopathy (FSE), chronic wasting disease (CWD) of deer and elk, and bovine spongiform encephalopathy (BSE). The potential zoonotic character has only been demonstrated for BSE with the discovery of a new variant of Creutzfeldt-Jakob disease (vCJD) in 1996 (5,13, 25).

Chronic wasting disease (CWD) has recently emerged in North America (NA) as an important prion disease of captive and free-ranging cervids (37). CWD is the only recognized transmissible spongiform encephalopathy (TSE) affecting free ranging-species. Three cervid species, mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), and Rocky Mountain elk (*Cervus elaphus nelsoni*), are the only known natural hosts of CWD. Endemic CWD is well established (1). Apparently CWD has also infected farmed cervids in several states, and has probably been endemic in North America's farmed deer and elk for well over a decade. The precise time and place of most emerging diseases origins cannot be determined with certainty; CWD is no exception. It was recognized for the first time in a free-ranging elk in Colorado in 1981 (29) but surveillance data and epidemic modeling suggest that CWD may have been present in some of the deer populations in NA for at least twenty years earlier (17).

Detection of CWD in captive and free-ranging cervids is based on various combinations of clinical observation and laboratory diagnostics that form the foundation for established surveillance strategies. Clinical observation remains a common tool for detecting CWD in both captive and free-ranging cervids. The most striking and widely recognized clinical features of final-stage CWD in deer and elk are behavioural changes and loss of body condition (33,34). Because clinical signs are neither consistent nor diagnostic, CWD diagnosis must be confirmed by examination of the brain for spongiform lesions (33,34,36) and/or accumulation of PrP^{CWD} (PrP^{res}) in brain and lymphoid tissues by immunohistochemistry (IHC) (17,18,20,29).

As with other animal TSE's, the public health

implications of CWD overshadow more tangible implications for the health of important wildlife resources. Despite media innuendo to the contrary, no cases of human prion diseases have currently been associated with CWD. Nevertheless, the increasing spread in the USA of CWD has raised concerns about the potential for increasing human exposure to the CWD agent. Recently, the conversion of the human prion protein by CWD-associated prions has been demonstrated in an in vitro cell-free experiment (1). However, since the basis of the transmission barrier in relation to the TSE is complex (10,24), more epidemiologic and laboratory studies are necessary to monitor the possibility of such a transmission under natural conditions. Whether transmission of the CWD prion among cervids requires direct interaction with infected animals has been unclear. Miller and others (19) reported that it could be transmitted to susceptible animals indirectly, from environments contaminated by excreta or decomposed carcasses and this after more than 2 years.

There are no reports of CWD in areas outside NA with the exception of a single animal imported into Korea from Canada (27). Available information indicates that there is only negligible trade in live cervids originating in NA to Europe but there are indications of imports of small annual tonnage of edible products for game. Additionally, it is unclear what, if any, trade exists in antler, embryos or semen from cervids between NA and European countries (24). Currently, only a few European countries conduct surveillance programs on TSE in free-living or captive cervids. Given that the possible risks of exposure relate to the tissues of cervids from NA, reinforced protection of the cervid population and animal and public health in Europe could be considered. Moreover, systematic surveillance is essential to establish the probability of occurrence and incidence of CWD in the *Cervidae* populations of Europe (10,24). In Belgium, wild ruminants are not screened for TSEs as part of a specific TSE surveillance scheme. Until 2001, the year that the WHO declared Belgium rabies-free (4,38), wild ruminants were checked in the follow-up of the rabies surveillance scheme of animals found dead in the wild or in parks (3). This resulted in a number of 56 wild cervids tested and found negative for TSE since 1990. This number is rather small and due to the rabies-free status, Belgium has lost this sole mean of testing this group of animals. Therefore, new means had to be found to have some kind of surveillance. In accordance with the opinion of the European Scientific Steering Committee and the European Food Safety Authority (EFSA), we have installed a preliminary surveillance scheme in order to estimate the true CWD prevalence of free-ranging cervids (*Capreolus capreolus* and *Cervus elaphus*) in the south-eastern part of Belgium.

MATERIALS AND METHODS

Population

Wild deer are mainly found in the Walloon region, particularly in the south-eastern part of Belgium. In this region, the population size of wild deer increased two-fold during the last two decades (7). Three species of cervids are currently present in the Walloon region. The size of the populations was estimated around at 40,000 head of *Capreolus capreolus* (roe deer), around 10,000 head of *Cervus elaphus* (red deer) and around 200 head of *Dama dama* (fallow deer), with respectively more than 95%, around 75% and more than 95% of those living in the south-eastern part of Belgium. (7). The estimated number is more precise for Capreolus capreolus (indicative value of $\pm 10\%$) than for the other species (6).

Sample sources and collection

A total of 866 hunter-killed wild cervids (674 *Cervus elaphus* and 192 *Capreolus capreolus*) from different hunting areas in the south-eastern part of Belgium was included (Table 1). All animals were necropsied and sampled during the hunting seasons 2001 to 2003. The spleen fragments used for the study were dissected within 2-3 hours, and frozen within 8-10 hours after death.

Testing strategy

The enzyme-linked immunosorbent assay (ELISA) (Bio-Rad, France) as rapid TSE screening test and the immunohistochemistry (IHC) as confirmation test were applied on each spleen sample.

Bio-Rad laboratory's ELISA test

The Bio-Rad TSE ELISA was used for purification and detection of PrP^{CWD}. A detailed assay procedure has been described in the instruction manual sent with the purification and detection kits. A brief description of the procedure of purification and detection is described by Hibler

	Red deer							Roe deer							
	Juvenile		Subadult		Adult			Juvenile			Adult				
Year	♂	♀	♂	♀	♂	♀	Subtotal	♂	♀	n.d.	♂	♀	n.d.	Subtotal	Total
2001	28	49	7	10	17	56	167	6	3	1	17	22	0	49	216
2002	76	68	14	21	25	88	292	11	16	0	17	14	0	58	350
2003	58	32	13	26	29	57	215	12	8	1	29	34	1	85	300
Total	162	149	34	57	71	201	674	29	27	2	63	70	1	192	866

Table 1. Composition of the wild deer sampling in the south-eastern part of Belgium according to the CWD surveillance. All animals were sampled during hunting seasons (October-December). Juvenile: <1 year. Subadult: 1-2 year (only red deer). Adult: >2 years for red deer and >1 year for roed deer. n.d.: not determined.

and others (12).

The principle of the test is based on the selective degradation of normal membrane-bound prion protein (PrP^{sen}) (16) by proteinase K (PK) treatment. The PK digestion step and centrifugation ensure selectivity of the test because PrP^{CWD} is resistant to proteolysis and co purifies with infectivity. The sample treatment procedures concentrate PrP^{CWD}, increase the sensitivity of the test. Measurements are made using the conventional 2-site sandwich ELISA because it provides more sensitive, more specific, and more rapid measurements than others assay formats. Monoclonal antibodies directed against PrP, by immunizing mice, are used for the detection of PrP^{CWD} (12)

Immunohistochemistry (confirmation test)

The technique used is described by Van Keulen and others (31) but using both a polyclonal (R524 - CIDC - The Netherlands) and 2 monoclonal antibodies, the 2G11 (Institut Pourquier - France) and the 12F10 (Spi-bio - France). As a positive control for IHC we used brainstem and lymphoid tissues of a Canadian CWD case.

Statistical analysis

The true CWD prevalence was estimated in a Bayesian framework, using WinBUGS 1.4. (28).

RESULTS

TSE detection using both ELISA and IHC

All samples were TSE negative using the ELISA (Bio-Rad) test and this was confirmed using IHC (with all 3 antibodies). The positive control used for IHC stained positive in both brainstem and in the lymphoid tissue for all antibodies, with a similar distribution pattern.

Estimation of the true prevalence of CWD according to the Bayesian framework

Choice of the prior information

The sensitivity and the specificity of the surveillance by the ELISA test and confirmation by the IHC test were estimated from data collected from the literature. IHC is still considered by most experts in the CWD field to be the "gold standard" (8,11, 14,22). It allows precise anatomical considerations of PrP^{CWD} deposition (8). However, a study, by Spraker and others (30) on 26 naturally infected mule deer in terminal stages of CWD (16 free-ranging and 10 captive) showed that there was only PrP^{CWD} detection in the spleen by IHC with in 53% of the free-ranging deer and 44% of the captive deer. We selected a Beta (alpha=12, beta=12) distribution (2.5^{th}-percentile = 0.31; median = 0.50; 97.5^{th}-percentile = 0.69) as prior information for the sensitivity of the IHC. Further, IHC is considered not to produce any false positive reactions (11). Consequently, the specificity of the IHC was fixed at 1. Comparing ELISA with IHC showed that ELISA was highly accurate: all animals with positive ELISA results were confirmed by IHC (24). The relative sensibility of ELISA depends on the species and tissue type and ranges from 0.92 to 1 (12). We selected a uniform (0.90-1) distribution as prior information. The relative specificity of the ELISA depends on the species and was 0.999-1 (12), so we selected a uniform (0.999-1) distribution as prior information.

Considering the age of the cervids, the animals less than 12 months of age were diagnosed with pre-clinical infection by IHC (14). The youngest elk diagnosed with clinical CWD was 17 months old (2,24,35). For this reason we did not consider the influence of the age in the estimation of the prevalence of the CWD in cervids.

Estimation of the true prevalence

Because of the high agreement between the ELISA test and the IHC test (12), it was assumed that results of the two diagnostic tests, which were used in this study, are dependant conditionally on the infection status of the tested animals. Based on a Bayesian approach and using the above prior information, the true prevalence of CWD in south-eastern part of Belgium was estimated to have a median value of zero with a 95% percentile value of $1.15\ 10^{-3}$.

DISCUSSION

The animal TSEs can be divided into two groups based on epidemiology and pathogenesis. The first group includes scrapie and CWD. Both of these diseases are characterized by the occurrence of lateral transmission, the ability to sustain the disease in populations, and widespread involvement of the lymphoid system early in the course of incubation. This first localisation of the CWD PrP^{res} reflects the initial oral pathway of CWD infection in cervids (26,37). The second group includes TME, BSE, and associated spongiform encephalopathies in felids and exotic ruminants infected with the BSE agent. In these diseases, epidemics are clearly related to ingestion of the agent in contaminated feedstuffs and host populations do not sustain epidemics. Involvement of lymphoid tissues appears to be limited or absent in this second group of animal TSEs (32).

In CWD-affected animals, infectivity is probably related to the highest prion concentrations in portions of the brain, spinal cord, spleen and lymph nodes (37). The relative levels of infectivity in these tissues are currently not available (2). The reliability of the spleen as a target tissue for detecting PrP^{CWD} by IHC in captive and free-ranging mule deer with clinical CWD was previously described (30). Additionally, the spleen also offers a more practical advantage. In fact, it is easier to convince hunters to give it up for scientific research than asking them for the head in order to sample the brain, as in most cases the heads of deer are a hunting trophy. The use of the ELISA technique, validated for brain tissue, on spleen tissue proved to be reliable for detecting TSE in sheep (9).

Currently, as there are no tests validated by the EU in order to evaluate TSE in European cervids, EFSA suggests that the techniques validated by the Food and Drug Administration can also be used but that they need to be confirmed by IHC (10).

Today, only a few European countries conduct surveillance programs on TSE in free-living and/or captive cervids and only a few experimental research studies are conducted to obtain data on the susceptibility of European cervids to TSE (10). Recently, German researchers (23) screened 849 free-ranging ruminants from Bavaria, including mainly roe deer and red deer. All samples were negative. In this region, the prevalence of the TSE was estimated to be below 0.5% for roe deer and below 1.5% for red deer with a 95% confidence. Additionally, preliminary CWD surveillance was performed in 136 Sika deer (*Cervus nippon*) in Japan (15) and all samples were also negative.

In addition, a retrospective epidemiological study of neurologically expressed disorders (NED) in ruminants in Belgium revealed that the positive predictive value of presumptive clinical diagnosis versus necropsy of NED in wild ruminants was low (13%) and can probably be explained by the low level of clinical observation and scarce anamnesis for wildlife (21). In this study, the percentage of NED cases where no etiological cause could be established for this same group of animals was high (77%). This finding suggests the necessity to install and maintain a active epidemiosurveillance network in wild cervids.

For the moment, we have only investigated samples originating from the south-eastern part of Belgium and the true prevalence of CWD in this region, estimated in a Bayesian framework, was found to have a median value of 0% with a 95% percentile value of 0.115%. This represents an improvement of our knowledge of the CWD status in wild cervids in the south-eastern part of Belgium. The data obtained are suitable for this part of Belgium but whether they are representative for the whole of Belgium remains unknown.

There are different reasons for maintaining, extending and improving this first CWD surveillance of the cervids population in Belgium: (1)more roe deer in the south-eastern part of Belgium should be sampled in relation to the population size, (2)currently, no wild roe deer samples (estimated number of 19,500 head) and no farmed cervids samples (estimated number of 13,000 head which are artificially fed during their live) from the northern part of Belgium were tested, (3)in

Belgium, as in many European countries, wild cervids are also artificially fed during the winter which results in frequent gatherings of the animals in these feeding areas. This management practice along with the known horizontal transmission of the CWD prion could dramatically favor the spread of CWD once the first cases develop, and [4]according to the early detection, the lymphoid tissues that drain the oral and intestinal mucosa are also possible samples for future investigations.

ABBREVIATIONS

BSE: Bovine spongiform encephalopathy
CWD: Chronic wasting disease
EFSA: European Food Safety Authority
ELISA: Enzyme-linked immunosorbent assay
FSE: Feline spongiform encephalopathy
IHC: Immunohistochemistry
NED: Neurologically expressed disorders
PK: Proteinase K
PrP: Prion protein
TME: Transmissible mink encephalopathy
TSE: Transmissible spongiform encephalopathy
vCJD: Variant of Creutzfeldt-Jakob disease

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