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First chronic wasting disease (CWD) surveillance of roe deer (*Capreolus capreolus*) in the Northern part of Belgium

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SUMMARY

Cases of chronic wasting disease (CWD) in wild cervids have yet not been reported in Europe, whereas the disease is considered enzootic in free-ranging mule deer, Rocky mountain elk and white-tailed deer in the area of Colorado, Wyoming, and Nebraska. New foci of CWD continue to be detected in other parts of the United States. However, no large-scale active epidemiosurveillance of European wild cervids is yet 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 wild cervids (roe deer) in the Northern part of Belgium. Spleen samples (n = 206) and brain samples (n = 222) of roe deer collected in the Northern part of Belgium, were examined for CWD using the antigen-capture enzyme-linked immunoassay (EIA) of IDEXX. Afterwards, the EIA was systematically confirmed by immunohistochemistry using three antibodies, namely R524, 2G11 and 12F10. There were no indications on the occurrence of TSE in any of the samples. A Bayesian framework was used for the estimation of the true prevalence of CWD in the Northern part of Belgium that was estimated to have a median value of zero with a 95th percentile value of 0.0049 and 0.0045 for spleen and brain samples respectively.

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), feline spongiform encephalopathy (FSE), chronic wasting disease (CWD) of deer and elk, and bovine spongiform encephalopathy (BSE). The potential zoonotic character is only demonstrated for BSE with the discovery of a new variant of Creutzfeldt-Jakob disease (vCJD) in 1996 (7,13,26).

Chronic wasting disease (CWD) has recently emerged in North America (NA) as an important prion disease of captive and free-ranging cervids (38). 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 (3). 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 CWD origins cannot be determined with certainty. It was recognized for the first time in a free-ranging elk in Colorado in 1981 (30), 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 (16). Very recently, two discoveries are dispatched: a moose killed in North-central Colorado has been tested positively for chronic wasting disease (11) and, by inoculating, transgenic mice expressing cervid protein with extracts of leg muscles from mule deer naturally infected with CWD (1). There are no reports of CWD in areas outside NA with the exception of a single animal imported into Korea from Canada (28).

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 (34,35). Because clinical signs are neither consistent nor diagnostic, CWD diagnosis must be confirmed by examination of the brain for spongiform lesions (34,35,37) and/or accumulation of PrP^{CWD} (PrP^{res}) in brain and lymphoid tissues by immunohistochemistry (IHC) (16,17,19).

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 (3). However, since the basis of the transmission barrier in relation to the TSE is complex (9,25), 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 et al. (18) 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.

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 (9,25). 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 (6,39), wild ruminants were checked in the follow-up of the rabies surveillance scheme of animals found dead in the wild or in parks (5). This resulted in a number of 56 wild cervids tested and found negative for TSE since 1990. 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*) in the Northern part of Belgium. A similar study has already been done for roe deer and red deer (*Cervus elaphus*) in the South-eastern part of Belgium (20).

MATERIALS AND METHODS

Population, sample sources and collection

Wild deer are mainly found in the Walloon region, particularly in the South-eastern part of Belgium (20). However roe deer are also present in the Northern part of Belgium, especially in the provinces Antwerp, Limburg and Vlaams-Brabant. In this late region, the size of the populations was estimated around at 19.500 head of *Capreolus capreolus*.
A total of 428 hunter- and or road-killed roe deer samples (222 brain samples and 206 spleen samples) were included in the survey. All animals were necropsied and sampled during 2005.

Testing strategy

The antigen-capture enzyme-linked immunoassay (EIA) technique of IDEXX (IDEXX HerdChek* Chronic Wasting Disease (CWD) Antigen Test Kit - IDEXX, The Netherlands) was used for the detection of the abnormal conformer of the prion protein (PrP^{sc}) as a rapid TSE screening test. Immunohistochemistry (IHC) was applied as confirmation test on each sample.

IDEXX HerdChek CWD Antigen Test Kit, EIA

The IDEXX EIA test was used for purification and detection of PrP^{CWD}. A detailed assay procedure is described in the instruction manual sent with the kit.
The principle of the test is based on a PrP^{sc}-specific ligand immobilized on the surface of the CWD antigen-capture plate, that allows detection of abnormal prions. Test samples are prepared by homogenizing the sample, followed by diluting the sample with working diluent. After the sample is applied to the plate, the disease-associated conformer binds to the immobilized ligand with high affinity. The plates are washed to remove unbound materials, including the normal conformer of the PrP protein. Following incubation with conditioning buffer, the captured antigen is then detected using a PrP-specific antibody that has been conjugated to horseradish

peroxidase. The plate is washed to remove unbound conjugate, and a peroxidase substrate is added. Color development is related to the relative amounts of PrP^{sc} captured by the ligand immobilized in the microtiter plate well. Interpretation of sample results is based on the absorbance of the sample. Samples whose absorbance are less than the cutoff value are classified as negative by the IDEXX kit. Samples whose absorbance are greater than or equal to the cutoff are classified as positive for PrP^{sc}. A confirmatory assay such as immunohistochemistry is required for all positive test results.

Immunohistochemistry (confirmation test)

The technique used is described by Van Keulen and others (32) but using both a polyclonal (R524 - CIDC - The Netherlands) and two 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. (2,29).

RESULTS

TSE detection using both EIA and IHC

All samples were TSE negative using the IDEXX EIA test and this was confirmed using IHC (with all three antibodies). However, due to the lack of fresh CWD positive material, fresh TSE positive ovine brain and spleen samples were included in the IDEXX EIA test. These samples all tested positively and served as an extra positive control. The positive control used for IHC stained positive in both brainstem and 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 EIA test and confirmation by the IHC test were estimated from data collected in the literature. IHC is still considered by most experts in the CWD field to be the "gold standard" (8,10, 14,23). It allows precise anatomical considerations of PrP^{CWD} deposition (8). However, a study, by Spraker and others (31) 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 in 53% of the free-ranging deer and 44% of the captive deer. For spleen samples, 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 (20). For brain samples, we selected a Beta (alpha=600, beta=94.77) distribution (2.5^{th}-percentile = 0.837; median = 0.864; 97.5^{th}-percentile = 0.888) as prior information for the sensitivity of the IHC. Further, IHC is considered not to produce any false positive reactions (10). Consequently, the specificity of the IHC was fixed at 1 both for spleen and brain samples.
Comparing EIA test with IHC showed that EIA test was sufficiently accurate: most of the animals with positive ELISA results were confirmed by IHC (9,22). The relative sensibility of EIA test is only available for lymph nodes of mule deer and white tailed deer and ranges from 0.944 to 0.984 (9). More generally, the relative sensitivity of enzyme linked immunoassays 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 both for spleen and brain samples. The relative specificity of the rapid tests depends on the species and was high (12). The relative specificity of the EIA was 0.995-0.9996 (9,22), so we selected a Beta (alpha=1000, beta=1.7) distribution (2.5^{th}-percentile = 0.995; median = 0.9986; 97.5^{th}-percentile = 0.9998) as prior information both for spleen and brain samples.
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 (4,25,36). 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 EIA test and the IHC test (12), it was assumed that results of the two diagnostic tests, which were used in this study, are dependant conditional 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 the

Northern part of Belgium was estimated to have a median value of zero with a 95th percentile value of 0.0049 and 0.0045 for spleen and brain samples respectively.

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 localization of the CWD PrP^{res} reflects the initial oral pathway of CWD infection in cervids (27,38). 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 (33).

In CWD-affected animals, infectivity is probably related to the highest prion concentrations in portions of the brain, spinal cord, spleen and lymph nodes (38). The relative levels of infectivity in these tissues are currently not available (4). 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 (31). 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 IDEXX CWD EIA technique, validated for cervid lymphoid tissue proved to be reliable for detecting TSE in sheep's brain and lymphoid tissue (spleen) and cervid's brain tissue as well.

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 (9). Recently, German researchers (24) did a screening of 849 free-ranging ruminants from Bavaria, including mainly roe deer and red deer. All samples were negative. Additionally, preliminary CWD surveillance was performed in 136 Sika deer (*Cervus nippon*) in Japan (15) and all samples were also negative. The 866 tested roe deer and red deer of the South-eastern part of Belgium (20) 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.

There are different reasons for maintaining, extending and improving this first CWD surveillance of the cervids population in Belgium: (i)more roe deer in Belgium should be sampled in relation to the population size; (ii)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; (iii)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; (iv)according to the early detection, the lymphoid tissues that drain the oral and intestinal mucosa are also pertinent samples for future investigations.

Abbreviations

BSE Bovine spongiform encephalopathgy
CWD Chronic wasting disease
EFSA European food safety authority
FSE Feline spongiform encephalopathy
IHC Immunohistochemistry
NA North America
NED Neurologically expressed disorders
TME Transmissible mink encephaloptathy
TSE Transmissible spongiform encephalopathy
vCJD New variant of Creutzfeldt Jakob disease

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