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Neuropathological survey reveals underestimation of the prevalence of neuroinfectious diseases in cattle in Switzerland

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ABSTRACT

Neuroinfectious diseases in livestock represent a severe threat to animal health, but their prevalence is not well documented and the etiology of disease often remains unidentified. The aims of this study were to generate baseline data on the prevalence of neuroinfectious diseases in cattle in Switzerland by neuropathological survey, and to identify disease-associated pathogens. The survey was performed over a 1-year period using a representative number of brainstem samples (n = 1816) from fallen cattle. In total, 4% (n = 73) of the animals had significant lesions, the most frequent types of which were indicative of viral (n = 27) and bacterial (n = 31) etiologies. Follow-up diagnostics by immunohistochemistry, PCR protocols and next-generation sequencing identified infection with *Listeria monocytogenes* (n = 6), ovine herpesvirus 2 (n = 7), bovine astrovirus CH13 (n = 2), bovine herpesvirus 6 (n = 6), bovine retrovirus CH15 (n = 2), posavirus 1 (n = 2), and porcine astroviruses (n = 2). A retrospective questionnaire-based investigation indicated that animals' owners observed clinical signs of neurological disease in about one-third of cases with lesions, which was estimated to correspond to approximately 85 cases per year in the adult fallen cattle population in Switzerland. This estimate stands in sharp contrast to the number of cases reported to the authorities and reveals a gap in disease surveillance. Systematic neuropathological examination and follow-up molecular testing of neurologically diseased cattle could significantly enhance the efficiency of disease detection for the purposes of estimating the prevalence of endemic diseases, identifying new or re-emerging pathogens, and providing "early warnings" of disease outbreaks.

1. Background

An estimated 80% of emerging viral infectious diseases in humans are zoonotic, meaning that they have primary nonhuman sources (Taylor et al., 2001), and about 50% of these diseases present with neurological symptoms (Olival and Daszak, 2005). Livestock plays a significant role in spreading pathogens and can act as reservoir and amplifier (Welburn et al., 2015). Causes of neurological diseases in farm animals, however, frequently remain unrevealed. In consequence, there is a risk that neurological diseases remain unreported despite potentially representing a severe health problem to animals, people in contact with those animals, and consumers of animal products (Welburn et al., 2015).

Two basic types of surveillance are used to manage and control diseases: active and passive surveillance (Thrusfield, 2007, pp. 180–183). Active surveillance relies on larger-scale laboratory testing for the detection of specific diseases. Examples of current active surveillance schemes for livestock in European countries are those for bovine spongiform encephalopathy (BSE) (Doherr et al., 2001), bovine brucellosis, and infectious bovine rhinotracheitis (Forschner et al., 1988). The disadvantages of such systems are the high costs of sampling, logistics, and laboratory diagnostic procedures, and restriction to a defined panel of known diseases. In contrast, passive surveillance is based on mandatory reporting of clinically suspicious animals. It is

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relatively inexpensive, but prone to underreporting in the context of low disease awareness and due to misinterpretation of clinical signs, as well as economic considerations (Thrusfield, 2007, pp. 169–187).

Passive surveillance for neuroinfectious diseases in cattle was very effective during the BSE epidemic in the 1990s and early 2000s in Switzerland (Heim et al., 1997; Seuberlich et al., 2010). However, reporting and diagnostic follow-up of neurologically diseased cattle have declined significantly with decreasing BSE case numbers (Boujon et al., 2016). Clearly, the efficacy of current surveillance systems needs to be improved for the early detection of new and re-emerging neuroinfectious diseases threatening animal and human health.

Neuropathological investigation serves a pivotal role in disease surveillance because it provides a broad view of changes in disease prevalence and morbidity and mortality rates. It is unbiased in the sense that it involves surveillance for lesions, rather than a specific pathogen, and it can direct further etiological investigations based on the lesions identified (O'Toole, 2010). Thus, it aids not only estimation of the prevalence of endemic diseases, but also the identification of new or re-emerging pathogens in the framework of "early warning systems" for disease outbreaks.

In the present study, we conducted an active neuropathology-based survey of cattle in Switzerland, targeting adult cattle that died on farm or were euthanized due to disease and did not enter the food chain (fallen stock). Our results show that about 4% of the fallen cattle had brain lesions indicative of neurological disease, among which the majority were consistent with encephalitis. Follow-up investigations by molecular testing and next-generation-sequencing (NGS) identified pathogens known to be associated with encephalitis, but also unexpected pathogens, the relevance of which for neurological disease in cattle needs to be established. These results provide a solid baseline for prevalence estimation and point to the value of neuropathological data in disease surveillance.

2. Materials and methods

2.1. Tissue samples

Samples for this study included medulla oblongata tissues collected between April 2013 and April 2014 from fallen cattle aged > 36 months in the framework of the statutory BSE surveillance program in three laboratories serving the eastern, western, and central regions of Switzerland (Fig. 1A). These laboratories conduct routine BSE screening, and the proportion of samples taken from each laboratory corresponded to the number of BSE tests conducted there. As a control group, brainstem samples from healthy slaughtered adult cattle were collected at two slaughterhouses in eastern and western Switzerland.

After the completion of BSE testing, the samples were shipped to the NeuroCenter, Division of Neurological Sciences, University of Bern, Switzerland. Each tissue sample was divided into two parts; a coronal section obtained at the level of the obex was formalin fixed (in 4% buffered formaldehyde) and paraffin embedded (FFPE), and the remaining tissue was stored at −20 °C.

2.2. Sample size calculation

All sample size calculations were performed with the online WinEpi tool (de Blas, 2006). Statistical comparison of frequencies of reported neurological signs in different sample categories was done by Fisher exact test ($p < 0.05$). Prevalences were calculated by dividing the number of cases by the sample size. The expected total number of cases per year in the fallen stock population in Switzerland was calculated by multiplication of the prevalence with the number of animals in the fallen stock population.

2.3. Geographic mapping and anamnestic data collection

Maps were created with GPS coordinates of the last holdings where animals were registered using Google Maps (Map data [©2016], GeoBases-DE/BKG [©2009]).

To assess the clinico-pathological correlation, questionnaires were sent out to the animal holders for every cow with histopathologic lesions in the medulla oblongata, and the same number of questionnaires was sent out for a random sample of cows without lesions. The farmers were not informed whether histopathological lesions were observed in their animals, in order to avoid reporting bias. Questions covered the cause of death, illness before death (and duration), clinical signs, previous examination by a veterinarian (and tentative diagnosis), and central nervous clinical signs of the other animals on the farm in the last year. The original questionnaires were in German and French. An English translated version is provided in Supplementary file 1.

2.4. Histopathology

FFPE tissue was cut into 5-µm-thick sections, mounted on glass slides, stained with hematoxylin and eosin, and examined by microscopy to detect the presence of lesions. Observed lesions were classified according to the VITAMIN D system (Vandevelde et al., 2012) using the following categories: (1) suppurative inflammatory lesions, (2) non-suppurative inflammatory lesions, (3) toxic-metabolic/degenerative lesions, (4) vascular lesions, and (5) mixed/other. These categories provide indications regarding the cause and etiology of disease. When more than one type of lesion was observed in the same sample, each type was recorded separately.

2.5. Immunohistochemistry

IHC for the detection of *Listeria monocytogenes* was performed according to a protocol established in our laboratory when lesions were histopathologically characteristic of listeriosis (Henke et al., 2015). For the detection of rabies virus, the same IHC procedure was applied, with the exception that the trypsin treatment was omitted and a mouse monoclonal anti–rabies nucleoprotein antibody was used (Theil et al., 1998).

2.6. PCR assays

For PCR assays, tissue samples weighing approximately 25 mg were cut from frozen brainstem samples and used for DNA and RNA extraction. DNA was extracted with the DNeasy blood & tissue kit (Qiagen) and RNA was extracted using Trizol reagent (Thermo Fisher Scientific), according to the manufacturers' instructions. The extracted DNA and RNA were stored at −20 °C and −80 °C, respectively. The quality of each extract was controlled with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific), and only DNA and RNA extracts with concentrations ≥10 ng/µl and absorption ratios 260/280 nm above 1.4 were used in further experiments.

Reverse-transcription polymerase chain reaction (RT-PCR) assays for BoAstV CH13 were performed with the OneTaq one-step PCR kit (New England Biolabs) using the MA2 and MA4 primers (Mittelholzer et al., 2003). A pan-herpes nested PCR assay was performed with degenerated consensus primer based on the method described by Ehlers et al. (1999), using a 12-min instead of 3-min incubation time at 95 °C for initial denaturation and a 20-s annealing and elongation time in the second PCR. qPCR assays were performed to detect the presence of OvHV-2 and BHV-6 using Path-ID qPCR master mix (Applied Biosystems), as described previously (Hussy et al., 2001; Kubis et al., 2013; Stahel et al., 2013; Wuthrich et al., 2016).

Quantitative RT-PCR assays for bornavirus and Flaviviridae were conducted with the AgPath-ID RT-PCR kit (Thermo Fisher Scientific) according to the manufacturer's instructions. We used the p24 forward