

Zoonoses Surveillance in Italy (2000-2009): Investigation on Animals with Neurological Symptoms

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1. Introduction

Zoonoses are defined by the World Health Organization (WHO) as “Those diseases and infections naturally transmitted between vertebrate animals and man” (WHO 1959) (Palmer et al., 1998). They may be caused by viruses, bacteria, including chlamidia and rickettsiae, fungi, protozoa, helminths and arthropods (Krauss et al., 2003), and transmitted directly (through contact with skin, hair, eggs, blood or secretions) or indirectly (by insect vectors and ingestion of contaminated food). Currently, 1415 pathogens for humans have been identified and of these approximately 61% (868) are agents of zoonoses, some of which manifest with neurological signs; 132 agents are also associated with emerging zoonoses (Asjo et al., 2007; Matassa, 2007; Taylor et al., 2001). Neurological zoonoses are widespread, especially in the developing countries where they are not even diagnosed in most cases.

Emerging zoonoses of recently identified pathogens are Lyme disease, cryptosporidiosis, West Nile disease, transmissible spongiform encephalopathy, and possible variants of the avian influenza virus, which have found new favourable conditions for spreading. In contrast, re-emerging zoonoses are well-known diseases considered as eradicated in a given country but recur with an exponentially increasing incidence, such as tuberculosis, leptospirosis, rabies (Matassa, 2007).

Pathogens are constantly evolving and spreading in different countries through animals that act as an asymptomatic reservoir and release pathogens into the environment (Krauss et al., 2003). Among these, wild animals, both mammals and migratory birds, play an important role.

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Neurological diseases include those caused by highly pathogenic neurotropic agents such as rabies viruses and opportunistic agents that may develop disease in the immunocompromised. These agents belong to the genus Rhabdovirus, Herpesvirus, Flavivirus, Alphavirus, Bornavirus and Circovirus; others are bacteria such as *Listeria monocytogenes*, *Borrelia garinii* and *Borrelia afzelii*, *Chlamydophila psittaci*, *Campylobacter jejuni*, or parasites such as *Toxoplasma gondii*, *Encephalitozoon cuniculi*, and *Halicephalobus gingivalis*. Recognized neurozoonotic agents among the fungi are *Aspergillus* spp., *Mucor* spp., *Candida* spp. and *Cryptococcus neoformans*.

Within the group of food-borne zoonoses, *E. coli* O157: H7 is a particularly relevant syndrome because ruminants, especially cattle, are the main reservoir of the bacterium, while milk and dairy products and meat are the vehicle of infection.

At a 2004 meeting on emerging zoonoses jointly organized by the WHO, FAO and OIE, the factors that contribute to the emergence of zoonoses were carefully analyzed: greater pathogen adaptation and resistance (new strains); increased drug resistance; increased susceptibility of humans and animals; climate change and so on.

Currently, there are about 60 (DPR 320/54 and European Directive 2003/99/EC), notifiable animal diseases in Italy, many of which are zoonoses, but this list is always likely to change. It is also worth pointing out that the existence of animal reservoirs (domestic and wild) and complex transmission mechanisms, by vector and food, make collaboration between human and veterinary medicine essential for ensuring public health safety.

The objective of this study was to investigate the presence of neurological zoonoses or zoonotic agents in Italy, and to estimate the epidemiological impact of neurological diseases potentially transmissible to humans. It is important to assess which species are at risk of disease and which are the best reservoirs for pathogens in order to understand the cause of their onset and which measures could be taken against their spread.

2. Materials and methods

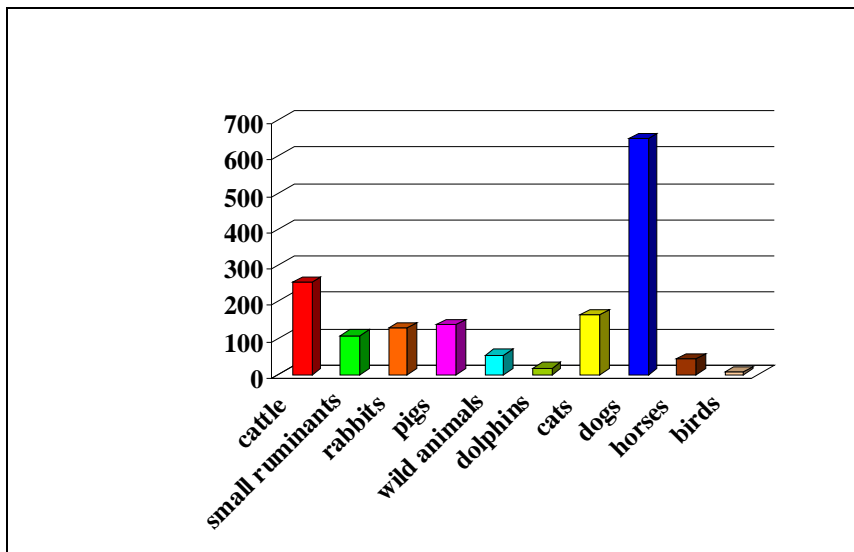
From 01 January 2008 to 31 December 2009, 990 animals of different species presenting with neurological signs were investigated in the different participating study centres.

All subjects underwent clinical examination and only cases with central nervous system (CNS) involvement were analyzed further. Serum or whole blood and cerebrospinal fluid of some animals (CSF) were collected and examined. When the animals died or were slaughtered, necropsy was performed and samples were collected from the various organs and the CNS. Depending on the symptoms reported, samples from the spinal cord, muscles and nerves were also taken. The tissues were frozen for cultural and/or molecular investigations and fixed in 10% buffered formalin for histological and immunohistochemical (IHC) analysis.

Moreover, a retrospective investigation of cases recorded in the database of the participating study centres from 01/01/2000 to 31/12/2007 was performed. The case history of 570 animals dead or slaughtered, presenting with neurological signs and inflammatory lesions was examined. Among these, all the cases had already been diagnosed as affected by a zoonotic disease and those with lesions related to inflammatory diseases potentially transmissible to humans were selected.

In order to identify the pathogenic agent, suspected cases were submitted to cultural, biomolecular and IHC investigation using frozen or formalin-fixed tissue and/or previously stored CSF.

A total of 1560 cases were studied in the period from 2000 to 2009 (Graph. 1).



Graph. 1. Graphic representation of different species of animals studied in the period from 2000 to 2009

Cerebrospinal fluid examination: CSF samples were collected from the cisterna magna or lumbar level. Quantitative determination of total protein was carried out by photometric colorimetric testing, using as a normal range for total protein 0-30 mg/dl for fluid from the cisterna magna and 0-45 mg/dl from the lumbar region. Cell count was performed by blood cell count using a Fuchs-Rosenthal chamber, taking 0-5 cells/ mcl as the normal range, while the sediment obtained by cytocentrifugation (600 rpm for 10 min) was read by light microscopy after Romanovsky-type staining (Diff Quick Stain).

Neuropathological examination: the CNS was divided by a paramedian cut in two parts including the cerebral hemisphere, cerebellum and medulla oblongata. Coronal sections of formalin-fixed brain were made at the level of the brain stem, cerebellum, thalamus, hippocampus, and basal ganglia, including the cerebral cortex and any other areas presenting with gross lesions.

To evaluate the histological lesions, each sample was processed, embedded in paraffin, and microtome sections of about 4-5 μ m were prepared; the sections were stained with haematoxylin-eosin. When necessary, specific histologic stains were also carried out, e.g., Masson's trichrome, Weigert van Gieson, Congo red, Luxol fast blue-cresyl violet (for evaluation of myelin), Bielschowsky silver-impregnation (for evaluation of axons), Gram staining, Grocott, Giemsa and Good-Pasture, periodic acid-Schiff's reagent (PAS) and Gomori trichrome, methenamine silver (for the recognition of fungal elements), mucicarmine (for recognition of *Cryptococcus neoformans*) and Ziehl Neelsen (for recognition of protozoan elements), as described in standard protocols (S. Daniel and T. Zanin, 1997). When the histological investigation did not allow a definite diagnosis, samples were subjected to IHC, cultural and molecular (PCR) examination.

Immunohistochemical (IHC) examination: on histological sections with suspected lesions, specific antigens for *Listeria* spp. *Encephalitozoon cuniculi*, *Streptococcus suis*, *Neospora caninum*, and *Toxoplasma gondii* were investigated. Antibodies from commercially available kits were used according to the methods specific for the antigen. Deparaffinized and rehydrated sections were subjected to different types of unmasking according to the protocols: microwave (750 W), trypsin (0.1% with 0.1% Ca carbonate) or in a bain-marie (citrate buffer pH 6.1 to variable temperature). After rinsing, the sections were incubated for 4-12 h at 37°C or overnight at 4°C with specific monoclonal antibodies diluted (1:50; 1:500; 1:1000) in PBS. Subsequent antibody detection was carried out using biotinylated goat anti-mouse or anti horse secondary antibody diluted (1:200-1:1000) in PBS, for 20 min at room temperature, followed by the avidin-biotin-peroxidase complex. (Vectastain ABC kit, Vector Laboratories). Immunoreactivity was visualized using 3,3'-diaminobenzidine as chromogen; the sections were counterstained with Mayer's haematoxylin counterstain.

Cultural examination: bacteria isolation provides a first incubation at 35-37° C for 24-48 h on selective (Demi-Fraser; Oxford agar; Mac Conkey) and non-selective culture medium (blood agar with 5% sheep blood). Biochemical identification of the isolated strain was carried out on pure culture with an API Rapid system.

Biomolecular examination: frozen brain biopsies were individually macerated in a laminar flow bench and total nucleic acids were purified using a NucliSENS® easyMAG® system (bioMérieux, Inc., Durham, NC, USA) according to the manufacturer's instructions. Formalin-fixed paraffin-embedded samples were initially deparaffinized using a xylene-based technique prior to nucleic acid extraction. All samples were tested for cellular adequacy and absence of PCR inhibitors by PCR amplification of the b-actin gene DNA (for DNA extraction) or cDNA (for RNA extraction) as previously described (Salata et al., 2009).

The samples were tested by real-time RT-PCR for West Nile Virus (WNV) and enterovirus-RNA detection using the oligonucleotide primers and TaqMan probe targeting the WNV E gene (Lanciotti et al., 2000) or the enteroviral genome 5' untranslated region (Donaldson et al., 2002). In the procedure used, the nucleic acid (about 60 ng) was combined with Superscript® One Step RT-PCR System reagents (Invitrogen Ltd, Paisley, UK), primers and probe, reaching a total reaction volume of 20 µl, and amplified in a LightCycler® 2.0 real-time PCR System (Roche Diagnostics S.p.A., Monza, Italy).

Real-time PCR assays were used to test for the presence of *Toxoplasma gondii* (Lin et al., 2000), *Borrelia burgdorferi* (Exner et al., 2003), *Listeria* spp. and *L. monocytogenes* (Rodríguez-Lázaro et al., 2004), and *Chlamydia* spp. (Yang et al., 2006) with some modifications. Briefly, the extracted nucleic acid (about 60 ng) was assayed with an ABI PRISM 7700 sequence detector system (Applied Biosystems, Foster City, CA, USA) in 25 µl of a PCR mixture containing 12.5 µl TaqMan universal master mix, 15 pmol of each primer, and 10 pmol of the probe under standard amplification conditions.

End-point PCR assays were used to assay all samples for Borna virus (Cotto et al., 2003), tick-borne encephalitis virus (Puchhammer-Stoeckl et al., 1995), herpesvirus (Rose et al., 1997) and fungi (Sutar et al., 2004). Reverse transcription, if necessary, was performed as described by Bergonzini et al., 2009. Thermal cycling conditions were: one cycle at 95°C for 10min and 40 cycles at 95°C for 30 s; 50-60°C for 45 s; 72°C for 1 min; and an additional cycle at 72°C for 10 min.

To detect herpesvirus, a set of degenerate PCR primers targeting the highly conserved DNA polymerase genes of the Herpesvirus family was used, while in the case of fungi the rDNA internal transcribed spacer sequences were targeted by primers. Subsequently, herpesvirus

or fungi typing was carried out by bi-directional sequencing of amplicons using the BigDye1 Terminator v3.1 Cycle Sequencing Kit on a 3100 Genetic Analyzer (Applied Biosystems). After alignment with the SeqScape v2.5 software (Applied Biosystems), the sequences were compared with the ones available in GenBank by using the NCBI BLAST tool.

Electron-microscopy: in suspected cases of Leishmaniasis, Protothecosis and Neosporosis, electron microscopical investigations were carried out for the morphological identification of the aetiological agents. Selected tissues were cut into small pieces of about 3 mm³ which were post-fixed with osmium tetroxide, dehydrated with ethanol and embedded in epoxy resin. Sections of 1 micron thick were cut by ultramicrotome, stained with toluidine blue and observed by light microscopy. Selected blocks were then sectioned in ultra-thin sections, stained with uranyl acetate and lead citrate and observed by electron microscopy.

Muscle biopsy: muscle biopsies were received fresh and wrapped in gauze moistened with saline solution and then were snap frozen by immersion in isopentane precooled in liquid nitrogen and stored at -80 °C. Eight µm-thick sections were stained and reacted with a standard panel of histochemical stains and enzyme reactions (H&E, PAS, ORO, Gomori trichrome, ATPase pH 4.3 and 9.8, NADH-TR, SDH, COX, non specific esterase).

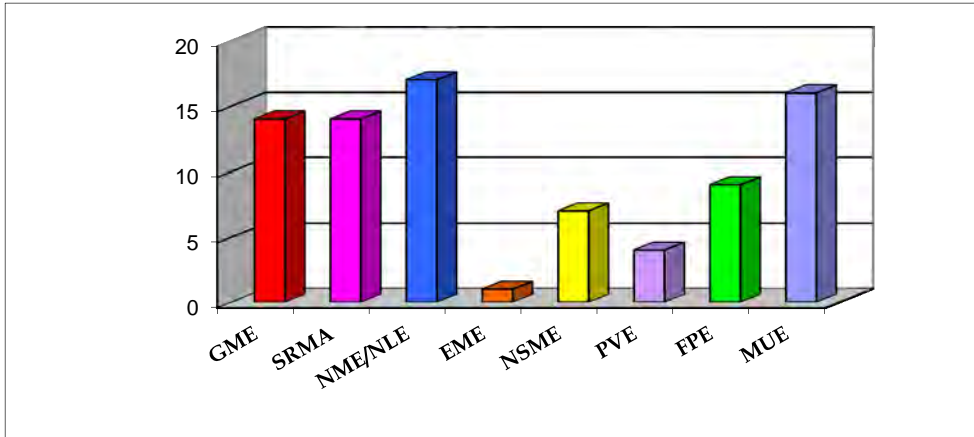
Nerve biopsy: nerve samples of 1 to 2 cm in length, depending on the nerve tested, were fixed in 2.5% glutaraldehyde solution in slight tension on a rigid support, to avoid artifacts of the nerve fibers. After washing in phosphate buffer, the samples were post-fixed in osmium tetroxide, dehydrated with ethanol of increasing concentration and embedded in epoxy resin. Sections of 1 micron thick were cut by ultramicrotome, stained with toluidine blue and observed by light microscopy.

3. Results

In 660 animals (42%), traumatic, vascular, congenital, muscular and articular diseases were diagnosed by clinical examination; no CNS involvement was found. In 353 subjects (23%), probable zoonoses were identified; 82 of these (73 dogs and 9 cats) showed neuropathological lesions associated with viral infections of unknown origin and therefore considered as "potential zoonoses" in the literature (Graph. 2). In 337 (22%) cases, pathologies not transmissible to humans were present: neoplasia, toxic-degenerative and bacterial diseases, distemper, FIV, FELV, FIP, IBR, CAEV Visna, border disease, etc., and therefore were not considered in this study. In 194 cases (12%), no diagnosis was established because the case history was incomplete and/or tissues were unsuitable for biomolecular investigations. Sixteen animals (1%) showed no lesions (Graph. 3). Overall, 16 zoonoses were detected in 271 animals of different species (Table 1).

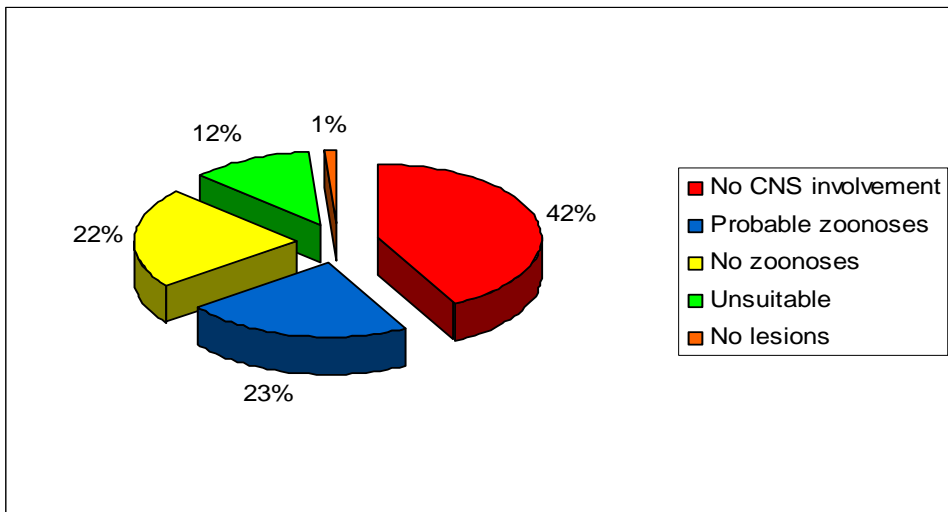
Encephalitozoonosis - In 110 (31.5%) rabbits, *Encephalitozoon cuniculi* was isolated. The clinical signs were variable, reflecting the site of lesions in the brain: convulsions, discoordination, paresis or paralysis and stiff neck were reported. The thalamus and hippocampus presented with more severe damage. In all animals there was a granulomatous meningo-encephalitis characterized by perivascular cuffs and focal granulomas composed of epithelioid histiocytes and lymphocytes, some of which showed a centre composed of amorphous eosinophilic material (Fig. 1). In all, 78% of these subjects were serologically positive for *Encephalitozoon cuniculi*; and in some cases IHC and Ziehl-Neelsen stain highlighted individual or aggregates of parasites within the granulomas, thus confirming the diagnosis (Figs. 2 and 3).

Listeriosis - In 47 (13.5%) cases (33 cattle and 14 sheep) *Listeria* spp. was isolated. Clinically, the animals showed depression, fever, paralysis, twisted neck, tremors, ataxia, motion



* GME: Granulomatous Meningoencephalitis; SRMA: Steroid-responsive meningitis-arteritis; NEM/NLE: Encephalo-mielitis/Leuco-encephalitis necrotizing; EME: Eosinophilic meningo-encephalitis; NSME: Non suppurative meningo-encephalitis; PVE: Periventricular encephalitis ; FPE: Feline polio-encephalomyelitis ; MUE: Meningoencephalomyelitis of undetermined etiology

Graph. 2. Graphic representation of diseases of "**unknown aetiology**".



Graph. 3. Graphic representation of results obtained in this study

ZOONOSES	CATTLE	SMALL RUMINANTS	DOGS	CATS	HORSES	PIGS	DOLPHINS	RABBITS	BIRDS	WILD ANIMALS
<i>Halicephalobiasis</i>					2					
<i>Neosporosis</i>	4		11							
<i>Borna disease</i>		2								
<i>Toxoplasmosis</i>			2	1		2	2			
<i>Rickettsiosis</i>			1							
<i>Nocardiosis</i>			1							
<i>Cryptococcosis</i>			3	1						
<i>Protothecosis</i>			1							
<i>Leishmania disease</i>			1							
<i>West Nile disease</i>					15					
<i>Unknown aetiology</i>			73	9						
<i>Listeriosis</i>	33	14								
<i>Colibacillosis</i>	15	1				5				
<i>Encephalitozoonosis</i>								110		
<i>Coenurosis</i>	1	16								
<i>Streptococcosis</i>						26				
<i>Chlamydia</i>	1									

Table 1. Zoonoses diagnosed in the period of study

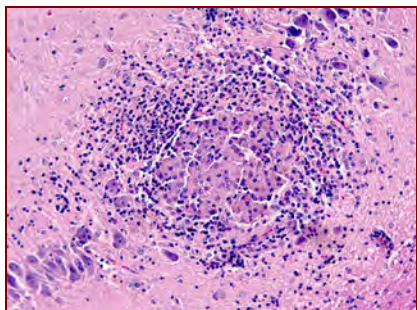


Fig. 1. Rabbit. *Encephalitozoon cuniculi*: parassitic granuloma in the hippocampus (HE, 20x).

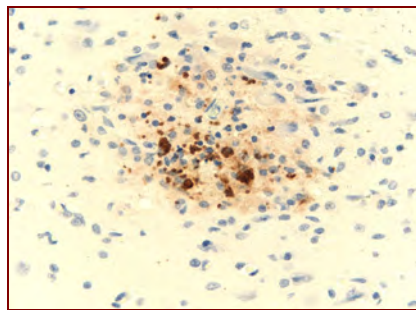


Fig. 2. Rabbit. *Encephalitozoon cuniculi*: parassitic antigen within a granuloma reaction (IHC, 40x).

handling, dysphagia, recumbency and death. The histological lesions showed a nonsuppurative meningo-encephalitis characterized by microabscesses (Fig. 4) and perivascular cuffings of lymphocytes and monocytes, distributed from the obex to the thalamus. The most severely affected areas were the pons and the midbrain, where often variable degree of haemorrhage was often observed. When fresh or frozen tissue (26 cases) was available, *Listeria monocytogenes* was isolated by cultural examination; moreover, IHC analysis showed the bacterial antigen predominantly in the microabscesses, less frequently in the perivascular cuffs (Fig. 5).

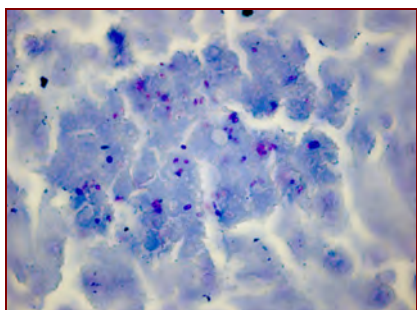


Fig. 3. Rabbit. *Encephalitozoon cuniculi*: parasites within a cerebral granuloma (ZN, 40x).

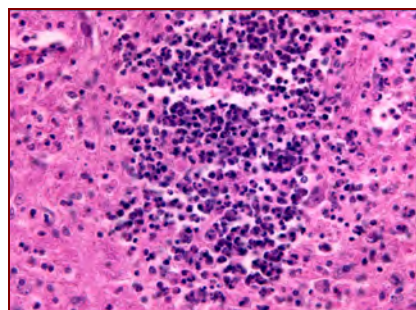


Fig. 4. Cow. *Listeria monocytogenes*: microabscess in the brain stem (HE, 40x).

Streptococcosis - In 26 (7.5%) pigs, a bacterial meningo-encephalitis was caused by *Streptococcus suis*. All our cases involved fattening piglets, 2 to 6 months of age, presenting with depression, anorexia, weight loss, ataxic gait, opisthotonus, and pedalling. The histopathological lesions showed a suppurative meningitis or mild to severe meningo-encephalitis (Fig. 6), diffuse lympho-histiocytic perivascular cuffing with some neutrophils, vasculitis, neuronal degeneration and haemorrhage; sometimes the lesions extended to the choroid plexus. In 14 cases, the bacterium was isolated directly from the CNS, and in 6 of these, also from other organs such as spleen, kidney, liver, lungs and trachea. Moreover, in

12 cases *S. suis* was found only at the level of the trachea and lungs, but only 6 of these had neuropathological lesions. IHC confirmed *S. suis* serotype 2 infection in 10 cases (Fig. 7).

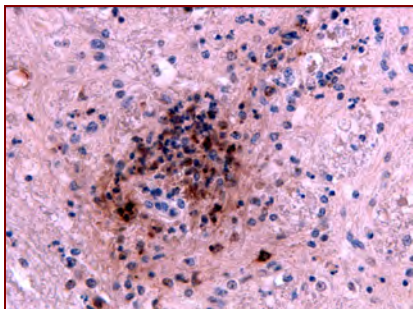


Fig. 5. Cow. *Listeria monocytogenes*: bacterial antigen within a microabscess (IHC, 40x).

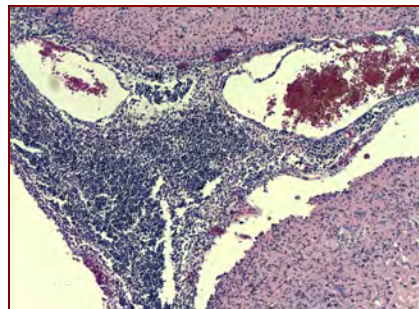


Fig. 6. Pig. *Streptococcus suis*: suppurative meningo-encephalitis (HE, 10x).

Colibacillosis - In 21 (6%) cases, involving 15 calves, 1 lamb and 5 piglets, *E. coli* was isolated. All presented with hyperacute or acute septicaemia, depression, ataxic gait, opisthotonus and sudden death. The neuropathological lesions were similar in all cases and showed a massive suppurative meningo-encephalitis characterized by mononuclear cells and a high amount of neutrophils. There was also marked vasculitis throughout the brain; in some cases, abscess-like lesions were visible, predominantly in the brainstem. The diagnosis was confirmed by bacterial culture.

Coenurosis - In 17 (5 %) cases (1 cattle and 16 sheep) larvae of the *Taenia multiceps* tapeworm were identified. Clinical signs and severity of the acute phase depended on the number of eggs ingested, the extent of inflammatory response, and the location of the parasite in the CNS. The clinical records reported weight loss, apathy, adynamia, tendency to isolation, depression, blindness, head tilt and ataxia. Histologically, focal haemorrhage or malacia caused by the migratory phase of the larval stage (acute stage), were observed at the level of the cerebral hemispheres or in the caudal brainstem. In the chronic phase, the visible cysts were surrounded by atrophic tissue. The diameter of the cysts, single or multiple, varied from 1 to 4.5 cm and appeared as an empty space surrounded by a granulomatous reaction composed of macrophages, giant cells, lymphocytes and plasma cells, and a few granulocytes. Outwardly, many macrophages, more or less extensive haemorrhage, fibrin or fibrin-purulent leptomeningitis and mononuclear perivascular cuffs were sometimes observed. In many cases it was possible to extract the parasitic cysts in which the cephalic protoscolices were visible.

West Nile encephalitis - West Nile virus was isolated in 15 (4%) horses. The horses were 9 females and 6 males, 2 to 19 years of age, mostly trotters from the provinces of Ferrara and Bologna. The horses underwent detailed neurological examination, blood-biochemical and serological tests. The blood-biochemical tests showed no significant changes, except in 5 horses in which there were slight alterations of inflammatory proteins in the acute phase. Three horses were asymptomatic, 12 had clinical signs attributable to acute forms such as dysorexia and depression, weakness, low-grade fever, hypersensitivity to touch and sound, ataxia of all four limbs (4 cases) or the hind limbs (4 cases), paraparesis (6 cases) or tetraparesis (3 cases), and three subjects presented with neurological signs indicative of intracranial involvement. In most cases (10 animals) these signs were mild to moderate and

quickly improved with recovery in all subjects. Only two horses were euthanatized because of severe disease progression; sampling of CSF, necropsy and histological examination of the CNS were performed. The first test showed moderate, predominantly mononuclear pleocytosis, and the histopathological examination revealed lesions distributed mainly at the caudal brainstem and the thoracic and lumbar spinal cord. In detail, a mild to moderate nonsuppurative encephalomyelitis characterized by perivascular cuffs of mononuclear inflammatory cells, glial nodules and focal gliosis in the gray matter were observed. In the spinal cord the lesions were bilateral and symmetrical, involving the gray matter, particularly the lateral and ventral horns (Fig. 8). At this level, a nonsuppurative inflammation was identified sometimes associated with neuronal degeneration, gliosis, neuronophagia and occasionally neutrophils. Small glial foci involved the white matter in both the spinal cord and brainstem; oedema and mild inflammatory infiltrate characterized by mononuclear cells were observed in the meninges.

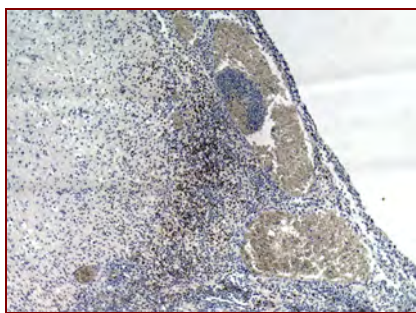


Fig. 7. Pig. *Streptococcus suis*: bacterial antigen within macrophages and neutrophils cytoplasm (IHC, 10x).

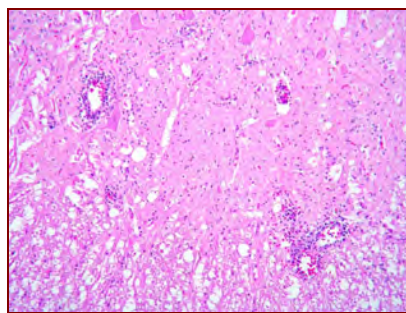


Fig. 8. Horse. *West Nile virus*: inflammatory infiltrate of mononuclear cells in the ventral horn of spinal cord (HE, 10x).

Neosporosis - In 4 calves and 11 dogs (4%) the protozoan *Neospora caninum* was found. The calves were a few days old and showed ataxia and limb paralysis. Histology showed similar nonsuppurative lesions in both the peripheral and the CNS. In detail, there were nonsuppurative meningomyelitis (meningo-encephalomyelitis in one case), polyradiculoneuritis, fibrosis of the peripheral nerves and neurogenic muscle atrophy. A large infiltration of lymphocytes and plasma cells was detected in the spinal meninges and nerve emergence of all spinal cord segments, especially at the level of cervico-thoracic and lombo-sacral intumescence. The inflammatory infiltrate appeared most abundant in the ventral roots along the perivascular spaces. In the most affected areas there was multifocal leucomyelitis and protozoan aggregates morphologically attributable to tachyzoites of the genus *Neospora*. In the spinal gray matter, a diffuse gliosis and chromatolysis of motor neurons were observed. The peripheral nerves showed Wallerian degeneration and marked fibrosis. The diagnosis was confirmed by PCR.

The dogs had paralysis, muscle weakness, ataxia, opisthotonus, head tilt and dysphagia. In 9 dogs the CNS was affected with meningo-encephalitis and myelitis; the other 2 dogs presented with polymyositis and chronic axonal neuropathy. An adult dog showed a severe cerebellar necrotizing inflammation with lymphoplasmacytic leptomeningitis, oedema of the white matter, incomplete atrophy and necrosis of the folia, with extensive loss of Purkinje and granular layer cells, often replaced by reactive astrocytes. In some areas, the

lesions were more severe, with necrosis, cavitation and the presence of gutter cells and haemosiderin pigment within macrophages. This nonsuppurative inflammation also extended to the subpial neuroparenchyma of the medulla oblongata and pons. The other brain areas were affected less frequently. Clusters of tachyzoites or protozoal cysts were found scattered within the lesions and sometimes in normal nervous tissue. The diagnosis was confirmed by electron microscopy and IHC (Figs. 9-10).

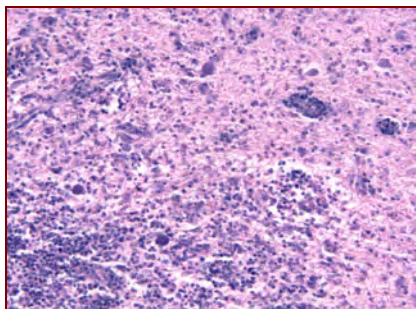


Fig. 9. Dog. *Neospora caninum*: granulomatous inflammation and protozoan cysts in cerebellum (HE, 0x).



Fig. 10. Dog. *Neospora caninum*: protozoan cysts react with specific antigen in cerebellum (IHC, 40x).

Toxoplasmosis - In 7 animals (2 dogs, 1 cat, 2 pigs and 2 dolphins) (2%) *Toxoplasma gondii* was isolated in the CNS. In the dogs, reported signs were fever, paralysis, vomiting and diarrhoea; the cat had nonspecific signs such as depression, anorexia and fever, vomiting, diarrhoea, prostration, swollen lymph nodes, ataxia, behavioural changes, and circling; the pigs showed ataxia, vomiting and diarrhoea. The histological lesions were typical of a subacute or chronic nonsuppurative meningo-encephalitis characterized by prominent perivascular cuffing of mononuclear cells and multifocal microglial nodules scattered in the surrounding neuropil. In the gray matter there were astrogliosis and mild to moderate neuronal necrosis characterized by vacuolation and chromatolysis. In the most affected areas (pons), lymphocytic vasculitis associated with haemorrhage, oedema and plasmorrhagia was predominant. Single or multiple roundish, basophilic toxoplasma cysts were found scattered in the tissue. The diagnosis was confirmed by IHC and RT-PCR. (Figs. 11-12).

Cryptococcosis - In 4 subjects (2 dogs and 2 cats) (1%) *Cryptococcus neoformans* was isolated. The cats presented with cutaneous nodules, nystagmus, mydriasis, seizures, and also nasal ulcers and sneezing in one case; the dogs showed only neurological symptoms, with seizures, ataxia and abnormal behaviour. The histopathological findings differed between the two species. In the dogs there was a granulomatous meningo-encephalomyelitis, the leptomeninges, brain and spinal cord showed marked multifocal granulomatous inflammatory lesions, with large numbers of macrophages, lymphocytes and plasma cells; in the cats, the inflammatory response was mild. In all cases there were numerous clusters of fungal bodies spread throughout the parenchyma, consisting of circular structures with a slightly eosinophilic core surrounded by a clear halo (capsule) lending it a typical soap bubble appearance, and some were in gemmating phase. The capsule was strongly mucicarmine and PAS positive. The gray matter showed a moderate gliosis. The diagnosis was confirmed by staining of histological sections with mucicarmine. (Fig.13).

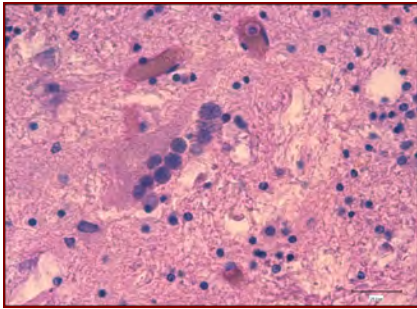


Fig. 11. Dolphin. *Toxoplasma gondii*: protozoan cysts in brain (HE,40x).

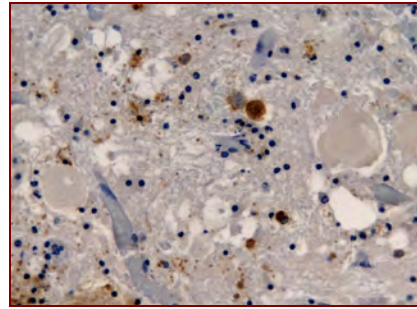


Fig. 12. Dolphin. *Toxoplasma gondii*: protozoan cysts react with specific antigen in brain (IHC, 40x).

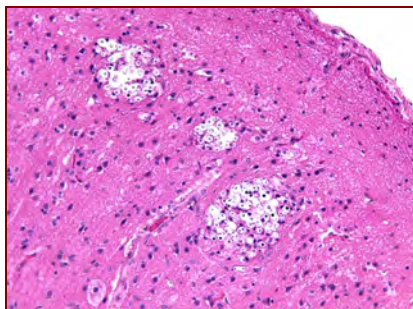


Fig. 13. Cat. *Cryptococcus neoformans*: packed yeast bodies with soap bubble appearance in the cerebral cortex (HE,10x).

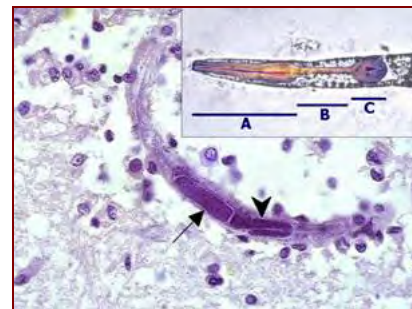


Fig. 14. Horse. *Halicephalobus gingivalis*: tangential section of a mature female in a necrotic area. Morphology of the rhabditiform oesophagus: A: body; B: isthmus; C: bulb (HE, 100x). From Mandara et al. Neuropatologia e neuroimaging, Poletto ed., 2011.

Halicephalobiasis - In 2 (0.5%) horses, *Halicephalobus gingivalis* was isolated and showed a multifocal neurological syndrome characterized by ataxia, circling, hyperexcitability alternating with depression and blindness in the left eye in one of two subjects. There was also stiffness, recurrent epistaxis and profuse sweating. Rectal temperature and clinical-biochemistry tests were normal. The neuroanatomical site of the lesion was diagnosed at the intracranial level. Neuropathological lesions were very similar. A granulomatous meningo-encephalitis extended predominantly in the cerebellum, brainstem and diencephalon and appeared to be characterized by perivascular infiltrates consisting of lymphocytes, plasma cells, numerous macrophages, multinucleated giant cells and scattered eosinophils. Parasitic elements, 10 to 15 µm in diameter, often oriented around the blood vessels, were present within the inflammatory lesions in the neuroparenchyma, meninges and subarachnoid space (Figs. 14 and 15).

Borna disease - In two sheep (0.5%) bornavirus meningo-encephalitis was diagnosed. The animals showed anorexia, ataxia, abnormal behaviour. The neuropathological examination

showed a severe nonsuppurative polioencephalomyelitis with perivascular cuffs of lymphocytes particularly around small-calibre veins of the frontal and temporal cortex, hippocampus, amygdala, and midbrain. At the hippocampal level there were also widespread gliosis with fibrillary astrocytes and neuronal necrosis (Fig. 16). The diagnosis was confirmed by real-time PCR and nested PCR.

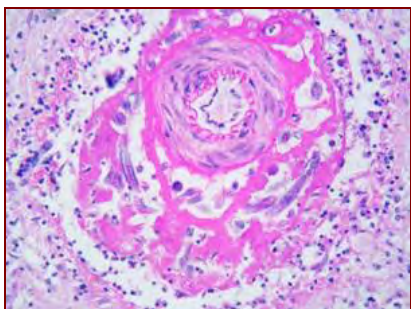


Fig. 15. Horse. *Halicephalobus gingivalis*: perivascular granulomatous infiltrate with presence of nematode around an arteriola (arrows) (HE, 40x)

From Mandara et al. Neuropatologia e neuroimaging, Poletto ed., 2011.

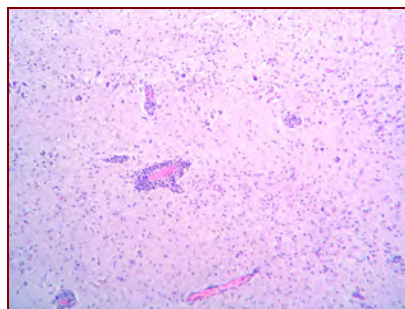


Fig. 16. Sheep. *Bornavirus*: perivascular cuffs and inflammatory infiltrate of mononuclear cells in hippocampus associated with pyramidal cells necrosis (HE, 4x)

Chlamydiosis - In a 6-year-old cattle (0.2%) bacterium of the genus *Chlamydia* spp. was isolated. The animal presented with abnormal behaviour, muscular tremors which increased when stressed, paralysis, weight loss, decreased milk production and corneal clouding. Histology showed a diffuse inflammation characterized by prominent lymphoplasmacellular and histiocytic perivascular cuffs, associated with vasculitis with few granulocytes. In the cortex there were neuronal necrosis, neuronophagia and marked gliosis (Fig. 17). The lesions were more pronounced at the level of the pons. The diagnosis was confirmed by real-time PCR.

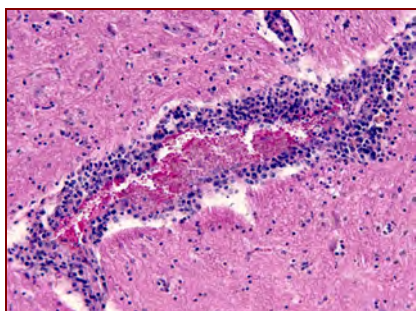


Fig. 17. Cow. *Chlamydia* spp.: perivascular cuffs of mononuclear cells (HE, 20x)

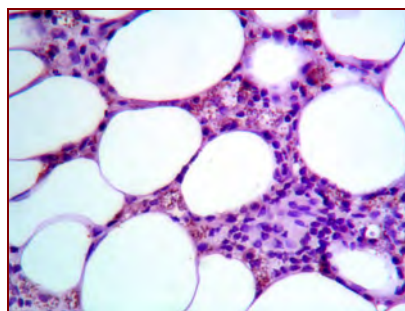


Fig. 18. Dog. *Leishmania infantum*: inflammatory infiltrate and numerous amastigotes in the epidural adipose tissue (HE, 40x)

Leishmaniasis - In one dog (0.2%) adult parasites of the genus *Leishmania* were isolated from a spinal granuloma in an extra-dural site compressing the spinal cord. The animal presented with lymphadenopathy and paraparesis. The lesion consisted of epidural adipose tissue infiltrated by inflammatory cells, primarily composed of macrophages, granulocytes, plasma cells and lymphocytes. In the cytoplasm of the macrophages, there was a large number of small spherical structures due to uniform amastigote forms of *Leishmania* spp. The diagnosis was confirmed by IHC (Fig. 18).

Nocardiosis - In one dog (0.2%) *Nocardia asteroides* was isolated. Macroscopically, there was an abscess-like lesion in the brain stem (Fig. 19). The dog showed fever, depression, difficulty walking and cranial nerve deficits. Histology revealed a piogranulomatous infiltrate consisting of neutrophils, macrophages and lymphocytes. Moreover, aggregates of club-shaped eosinophilic bacteria were found. The diagnosis was confirmed by bacterial culture.

Protothecosis - In one dog (0.2%) achlorophilic algae were isolated. The reported signs were gastrointestinal bleeding with chronic diarrhoea, depression, anorexia, and difficulty walking. Histology showed granulomatous lesions in the neuroparenchyma and meninges consisting of numerous macrophages, lymphocytes, plasma cells and some eosinophils. In the granulomatous foci there were numerous round bodies of variable size surrounded by a PAS positive capsule (Fig. 20), which were identified as achlorophilic algae of the genus *Prototheca*. The diagnosis was confirmed by electron microscopy.

Rickettsiosis - In one dog (0.2%) a microorganism of the genus *Rickettsia* spp. was isolated. The reported signs were fever, depression, lethargy, tetraparesis, ataxia, hyperesthesia, vestibular syndrome, seizures, and petechiae in the mucous membranes. Microscopically, there were a nonsuppurative meningo-encephalitis and vasculitis characterized by lymphohistiocytic-type cells and plasma cells infiltrating the nervous tissue and meninges; necrotic areas were also observed (Fig. 21). In this case the diagnosis was confirmed by PCR.



Fig. 19. Dog. *Nocardia asteroides*: abscessual lesion in the brain stem.

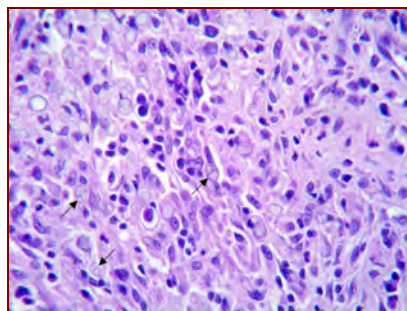


Fig. 20. Dog. *Prototheca*: numerous algae with a periodic acid-Schiff (PAS) -positive (arrows) (PAS, 40x)

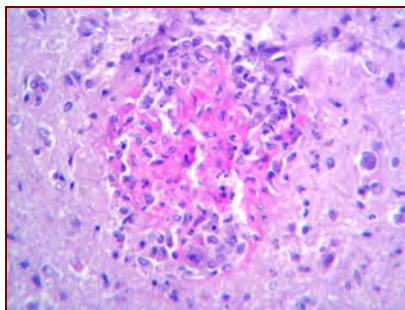


Fig. 21. Dog. *Rickettsia*: mononuclear vasculitis and fibrinoid necrosis of cerebrale arteriola (HE, 40x)

4. Discussion

In this study we investigated and analyzed the prevalence of neurological zoonoses in Italy in the decade between January 2000 and December 2009. A total of 16 different zoonotic diseases were diagnosed in 271 subjects of different animal species: 7 diseases were caused by parasites, 6 by bacteria, 2 by viruses and one case was due to achlorophilic algae. Farm animals were the species most affected; no diseases were found in birds or wild animals.

In 82 (23%) cases involving dogs and cats, the disease was of "**unknown aetiology**". Such inflammatory diseases are prevalent in the dog, with a clear preference for breed and sometimes with family involvement. In our study, the species most affected was the dog (Graph.2). These disorders may, in fact, be triggered by an as yet unidentified pathogen and are therefore to be considered "potential zoonoses" (Schwab et al., 2007). The histological lesions were often suggestive of viral infections, but no causative agent has ever been isolated so far. Both rabies and canine distemper have been suggested as possible causes, presumably as expressions of atypical infection with these viruses (Summers et al., 1995). New diagnostic techniques may be useful to identify the pathogens responsible for these diseases and assess their possible zoonotic risk.

In the group of defined zoonoses, **Encephalitozoonosis**, a parasitic disease, was found in 86% of the rabbits investigated (110 out of 128) coming from different provinces in Piedmont. *Encephalitozoon cuniculi*, an obligate intracellular parasite of microsporidia, can affect a wide range of mammals: rabbit, dog, cat, horse, fox, and humans as well. In the rabbit it usually presents as a chronic asymptomatic disease; in other species, especially in immunocompromised humans, more severe infections can lead to death (Mertens et al., 1997). The transmission route is probably by ingestion of food or water contaminated by spores shed with the urine of infected rabbits. Some authors suggest a vertical transmission in lagomorphs (Giordano et al., 2005). The disease is more common in pet rabbits (37-68%) than in the wild (Kunzel et al., 2010). In our study, 104 affected rabbits belonged to the group of meat animals, and only 6 were pets. Cases of human infection by microsporidia have been reported worldwide, but the natural reservoirs and modes of transmission remain to be elucidated (Furuya, 2009). *E. cuniculi* ranks third among the microsporidia that affect humans, after *E. bieneusi* and *E. bowel*, and it is also the cause of rare disseminated microsporidiosis forms, as described in a recent case of a woman with AIDS in Italy (Tasone et al., 2002). Many authors believe that the reported data are underestimated. Infection control in rabbit-breeding is very important and should not underestimate the risks to food-

industry workers. While the disease in rabbits is potentially transmissible to humans, it represents a significant risk only for individuals with severe immune system impairment. Didier et al. have identified three *E. cuniculi* strains (I rabbit, II mouse, III dog) based on the number of repeated sequences in the ribosomal internal transcribed region. All confirmed cases in humans since 1994, related to immunocompromised patients, mainly HIV-seropositive, were due exclusively to the dog and rabbit strain (Weber et al., 1997; Kunzel et al., 2010). It would therefore be interesting to analyse the cases described in this study with accurate biomolecular investigation in order to determine which strain they belong to.

Listeriosis, an infectious bacterial disease caused by *Listeria monocytogenes*, accounted for 13.5% of the zoonoses reported here. The infection is transmitted mainly through the consumption of contaminated food, but also by direct contact with infected animals. Compared to other food-borne diseases, it has a low incidence and high mortality rate in invasive neurological forms (20-30%) (Matassa, 2007). *L. monocytogenes* is a neurotropic bacterium and is more efficient than other neuroinvasive Gram-positive bacteria, including those of the *Streptococcus* genus, and it is one of the most common causes of bacterial meningitis in North America and Western Europe (Drevets et al., 2008).

Immunocompromised individuals, diabetics, alcoholics, the elderly and children can develop an invasive, often fatal form that is characterised by meningitis, septicaemia, endocarditis, pneumonia, arthritis, osteomyelitis and hepatitis; in pregnant women it can cause abortion (Matassa, 2007; Ramaswamy et al., 2007). In our study, as reported by other authors, we found a higher prevalence of the disease in ruminants as compared with other domestic mammals, suggesting that these species may constitute an important source of human infection, given the variety of foods derived from them (Oevermann et al., 2008). An epidemiological human study detected the presence of the bacterium in the stool of asymptomatic healthy subjects, but further studies are needed to understand the impact of these subjects on transmission (Drevets et al., 2008). Listeriosis is now considered an endemic infection in France and northern Europe (with peaks during the summer), unlike in Italy where it occurs sporadically (Bianchi et al., 1995). Recently reported human cases in Italy are: Genoa (n=1) (Viscoli et al., 1991); Monza (n=2) (Bianchi et al., 1995); Milan (n=1) (Ponticelli et al., 2005); and Naples (n=1) (Reynaud et al., 2007).

Among the pigs investigated in this study, **Streptococcosis** was diagnosed in 26 (7.5%) cases, all involving very young animals from fattening farms. The disease is caused by *Streptococcus suis*, a Gram positive bacterium, facultative anaerobic, which persists in the tonsils, sinuses, reproductive and digestive systems and can cause severe forms of meningitis, endocarditis and septicaemia. Piglets become infected after contact with colonized sows, which are healthy carriers; the infection can be transmitted to humans through direct contact with symptomatic animals or carriers or through ingestion of contaminated meat (Wertheim et al., 2009).

Higher rates of infection are attributed to stressing factors, poor hygiene and/or concurrent disease. *S. suis* has been isolated from other animals such as ruminants, dogs, cats, horses and wild animals, and it is believed to be a commensal in the intestinal flora (Staats et al., 1997). According to the polysaccharide capsule, 35 serotypes have been identified, including serotype 2 which is the main cause of infections in humans. The first human case was reported in 1968 in Denmark (Arends et al., 1988); since then, hundreds of cases have been reported around the world, many of which fatal (Lun et al., 2007; Wertheim et al., 2009). These multiple and severe outbreaks of *S. suis* in humans have raised public concern about its role as a zoonotic agent. Human infection manifests as a purulent meningitis, fever,

vomiting, dizziness, ataxia, petechiae, muscle pain, paralysis and hearing loss; sometimes toxic forms may develop, with septic shock leading to death (Lun et al., 2007). The histological lesions in humans resemble those found in pigs. The infection typically affects men between 47 and 55 years of age and is considered an occupational condition or it may be linked to particular dietary habits of a population. Previous studies using serological tests and nasopharyngeal swabs have found low positivity to serotype 2 *S. suis* in high-risk individuals such as veterinarians and pig farmers, confirming the role of humans as healthy carriers. Its prevalence, duration and importance in the spread of the disease remains to be investigated (Elbers et al., 1999; Wertheim et al., 2009). In this population subset, it probably behaves as an opportunistic pathogen that causes symptomatic lesions in special circumstances such as stress, AIDS and cancer. In Western countries the disease occurs only sporadically; in Italy three cases have been reported to date: one in a subject with neoplasia (Manzin et al., 2007), the other two were due to occupational exposure in a farmer and a butcher (Perseghin et al., 1995; Camporese et al., 2007). Most likely, prevalence data are underestimated because in its early stages of infection the disease is very often mistaken for a simple flu that resolves with generic antibiotic therapy in immunocompetent individuals. The difficulty of determining the true incidence of infection on farms makes real risk assessment problematic. For this reason it is important to maintain biosafety standards on farms and to consider potential *S. suis* infection in cases presenting with neurological signs.

In 17 (5%) ruminants we diagnosed **Coenurosis**, an infection caused by the larval stage of *Taenia multiceps*, a flatworm that develops in the intestine of canids (definitive hosts) which release hundreds of eggs with the faeces. Ruminants are the intermediate hosts, and the oncospheres often spread via the bloodstream in the body with possible CNS involvement. Normally, it is a solitary lesion involving one hemisphere and clinical signs vary depending on the location (Scott, 2000); during their migration the larvae may carry bacteria causing bacterial meningoencephalitis (Christodouloupoulos, 2007).

Only very rarely are humans affected; most cases have been reported in the developing countries (Africa and Asia) with a high mortality rate (Crusz, 1948; Fain, 1956; Raper and Edockera, 1956; Hermos et al., 1970). In Europe coenurosis in sheep has been reported in England, Ireland and France; in Italy it is mainly present in Sardinia, Latium, Puglia and Sicily. Man is considered an accidental dead-end host, where the infection most often develops in nervous, muscular and subcutaneous tissues. The first case of human coenurosis dates to 1913 (Brumpt, 1913); to date only a few hundred human cases have been described (Ing et al., 1998; Scala et al., 2006), some of which occurred in Italy (Sabbatani et al., 2004), at least five of which in Sardinia (Turtas et al., 1989).

In the CNS the parasite is localized predominantly in the submeningeal cortex, creating cysts that grow and compress the surrounding tissue, but it has also been found within the cerebral parenchyma and the spinal cord (Ing et al., 1998; Beniflá et al., 2007). In the Italian cases the cysts were generally located in the cerebral hemispheres in an eccentric position, and more rarely in the cerebellum. Several factors may increase the spread of the disease in sheep: illegal slaughtering, high number of dogs on the farm, and inappropriate use of anthelmintics (Scala et al., 2006).

Colibacillosis was confirmed in 15 calves, 1 sheep and 5 pigs (6% of total), all under 2 months of age. *E. coli* is a bacterium that usually lives in symbiosis with animals and humans, colonizing the gut, but some strains have developed virulence through the production of toxins or attachment factors. In particular, *E. coli* O157: H7 causes severe haemorrhagic enteritis and the haemolytic uremic syndrome (HUS), a disease that has

recently raised concern for the health of infants. HUS affects mostly very young children and the elderly and is characterized by acute renal failure and haemolytic anaemia; the mortality rate is 5%.

A serious complication, more common in adults, is thrombotic thrombocytopenic purpura which produces neurological signs. The syndromes caused by *E. coli* O157: H7 verocytotoxin belong to both the food-borne diseases and zoonoses because ruminants, particularly cattle, are the main reservoir of the bacterium. Milk, dairy products and undercooked meat carry the infection; human transmission is also very common (Matassa, 2007).

In adult cattle the course of infection is asymptomatic, except for cases of necrotizing mastitis in dairy cows. In calves, as occurred in the cases we report here, very serious septicaemic forms may appear starting from the earliest days of life, whereas lambs and piglets are less likely to be affected. In Italy the first case of *E. coli* O157 infection was reported in 1988. Since notification of the disease is not required and all reports are made on a voluntary basis, the final data are certainly underestimated. The most affected regions are Lombardy, Piedmont, Latium and Puglia. As of 2004 there were 439 reported cases of HUS, the majority of which occurred in children between 0 and 6 years of age (80%) (Matassa, 2007).

West Nile Disease (WNV), a zoonoses caused by a flavivirus (RNAvirus), is transmitted by mosquitoes of the genus *Culex*. In this series it was found in 15 horses (4%) and probably it is underestimated. Host amplifiers of the virus are migratory wild birds. The disease has seasonal trends and is more common in wetlands and marshy areas or near rivers, along the route of migratory birds (Castillo-Olivares et al., 2004).

The virus can infect several species of vertebrates (mammals, birds and reptiles); humans and the horse are accidental dead-end hosts because viraemia is short and does not favour the spread. In the horse, the course of the disease is often asymptomatic or mildly symptomatic, rarely is it fatal. In our series, 10 horses had mild clinical signs and recovered, three were asymptomatic but sero-positive, and only two showed severe signs and were slaughtered. In humans, it appears as a influenza-like syndrome, and only 1% of cases are fatal.

WNV in Italy was first reported in Tuscany in 1998 (Cantile et al., 2000); it then reappeared 10 years later around the Po Delta in 2008, where the virus resulted phylogenetically very close to that found in Tuscany where the disease is now endemic. In July 2009, the infection recurred as in 2008 but then spread to other regions. Since 2002, the Ministry of Health has established a national surveillance plan, both active and passive, to control the introduction and circulation of the virus and insect vectors in areas considered at risk.

The viral agent may induce a variety of clinical signs depending on the subtype and its geographic distribution (Gubler, 2007; Zeller et al., 2004). In our cases, the course of disease was short, evolving toward rapid improvement; neurological symptoms may sometimes go unrecognized and show only nonspecific signs. Therefore, the presence of asymptomatic or mildly symptomatic horses could lead to underestimation of the spread of disease. The WNV is a notifiable zoonosis, potentially fatal for humans, especially in the elderly, children and immunocompromised; therefore, monitoring of virus circulation in the country and wetland reclamation are important for reducing insect vectors.

During our investigations, **Neosporosis** due to *Neospora caninum* was found in 15 (4%) subjects (4 cattle and 11 dogs). It is a protozoan of the family *Sarcocystidae*, whose life cycle is not yet clear; tachyzoites and cysts are the only stages of the cycle identified to date. The presence of cysts has been described only in nervous tissue (brain, spinal cord, nerves and

retina), except for a solitary cyst in the eye muscle. The oocysts of the parasite have been isolated only from the faeces of dogs, which is considered to be both a definitive and intermediate host (Dubey et al., 1996; Cantile et al., 2002).

It can be transmitted both orally and vertically; in cattle the infection is feared especially because of severe economic losses due to its effects on reproduction, such as embryo absorption, abortion, return to heat, infertility and increased neonatal mortality with severe CNS infection in the calf (Dubey et al., 2003). In this study we observed lesions compatible with congenital or neonatal infection in calves, prevalently CNS lesions in adult dogs, and peripheral nervous system lesions (poliradiculoneuritis) in young subjects, as reported elsewhere (De Meerschman et al., 2005; Cantile et al., 2002). Although some authors have claimed no zoonotic role for *Neospora caninum* (Dubey, 2003), there is recent evidence that the protozoan may be responsible for neurological syndromes in immunocompromised HIV-seropositive individuals, causing opportunistic infections with *Toxoplasma gondii* (Lobato et al., 2006).

In 2% of cases **toxoplasmosis** was diagnosed, a parasitic infection caused by *Toxoplasma gondii*, an intracellular protozoa of the genus *Toxoplasma*, which affects several species of birds and mammals, including humans. The cat is the definitive host that spreads the oocysts into the environment through faeces, and other animals become infected through contaminated food and water. *Toxoplasma* can cause subclinical or clinical infection.

In our study all cases presented with neurological signs, one of the dogs also showed a concomitant infection with *Neospora caninum*. In domestic animals the disease generally has a very low prevalence, but it can represent a real risk of transmission to humans directly or through food. In pigs the reproductive (still-born and premature births) and respiratory (pneumonia) systems are most often affected, only rarely have myocarditis and encephalitis been reported, with a mortality rate of over 50% (Dubey et al., 2008). Among poultry, toxoplasmosis is very rare, mainly occurring in family-owned farms (Goodwin et al., 1994), while symptomatic toxoplasmosis in the horse has not yet been reported to date, although the infection is possible.

Sheep and goats have a high rate of abortion and still births, but there have been reports of lambs seven months of age with numerous cysts distributed throughout the carcass after slaughter (Dubey et al., 1989). In cats, as in humans, most infections are asymptomatic and rarely involve other organs or the CNS, as in kittens with congenital infection or immunodeficiency (FIV-FelV) (Davidson et al., 1993). In dogs the disease is more severe in pups; the most frequent signs are pneumonia, hepatitis and encephalitis. The two dolphins analyzed in this study were from strandings that occurred between 2007 and 2009 along the coast of Liguria (Italy).

This fact is of considerable importance for studying the pathogen's epidemiology and for the safety of public health and cetaceans. Many authors believe that toxoplasma may have infected the sea through the discharge of sewage along the coastline or ship ballast waters contaminated by onboard rodents, cats, or contaminated soil.

In this way, the dolphins may have been infected following ingestion of contaminated water and fish. The prevalence of this disease in animals is probably underestimated: the data are few and often not comparable because of differences in methods and the types of samples analyzed. One third of the human population is seropositive against *T. gondii* and this seroprevalence makes it an important agent of zoonosis. EC Directive 99/2003 provides that Member States report, depending its epidemiological status, all cases of disease and seropositivity detected, until a common control and surveillance system has been adopted.

Cryptococcosis is an occasional systemic infection caused by *Cryptococcus neoformans*, an opportunistic yeast-like fungus of the genus *Cryptococcus*. This disease is rare among domestic animals except dogs and cats; in fact, these two species were the only ones involved in our study. The most frequently isolated species are *C. neoformans* (serotype AD) and *C. gattii* (serotype BC). The latter are considered the most aggressive serotype (Lacaze et al., 2002). Pigeons are considered the main vector because their high body temperature protects them from disease. In immunocompetent birds cryptococcosis remains confined to the upper airways and sinuses because it prefers temperatures below 40 °C. The cat is the species most often affected and the most common route of infection is through the respiratory system; often, as in dogs, it also involves the eye and the CNS (15% of cases) (Berthelin et al., 1994). In natural conditions the immune response in humans provides resistance to infection, but in 85% of cases cryptococcosis affects the lungs and the CNS in debilitated patients within settings such as AIDS, tuberculosis, leukaemia and lymphoma (Summers et al., 1995; Kerl, 2003; Chuck et al., 1989; Nagrajan et al., 2000; Fernandes et al., 2000). *C. gattii* has also been reported in association with skin lesions in patients undergoing prolonged corticosteroid therapy (Bellissimo-Rodrigues et al., 2010). Even in the cat prolonged corticosteroid use may aggravate the infection, but there are no data demonstrating whether immunosuppressive diseases such as FIV and FeLV may predispose individuals to cryptococcosis (Gerds-Grogan et al., 1997; Kerl, 2003). In our cases, histopathological lesions were similar to those described previously (Summers et al., 1995). The cats showed low to moderate nonsuppurative inflammatory response, despite wide dissemination of the cyst both in the meninges and the neuroparenchyma; the response in the dogs was much more cellular, with granulomatous lesions and epithelioid macrophages, lymphocytes and plasma cells in the brain and meninges.

Halicephalobus gingivalis, a nematode which lives in soil and decaying plant material, was identified in two case of equine meningo-encephalitis. The parasite is pathogenic in the horse and man and infection occurs through skin injuries with spreading of the worm to other organs, including the CNS. In our cases *H. gingivalis* was found in two horses, one from Tuscany and the other from Veneto. In the horse the pathology is characterized by disseminated granulomas in the skin and other organs; granulomatous encephalomyelitis is often reported. In humans the infection is always fatal and affects the CNS (Gardiner et al., 1981). In our series only the CNS was involved and the histological lesions were similar to those described elsewhere (Mandrioli et al., 2002). The parasite was first fully described by Stefanski in 1954, from a gingival granuloma in a horse in Poland (Anderson et al., 1982). Four cases in horses have been reported so far in Italy, all native animals which apparently had never left the country, confirming the presence of the parasite in Italy (Cantile et al., 1997; Mandrioli et al., 2002). Worldwide, only three humans cases with fatal outcome have been reported, in all of which the CNS was involved (Akagami et al., 2007; Gardiner et al., 1981; Shadduck et al., 1979), but no human case has been reported in Italy so far.

Borna disease was diagnosed in 2 sheep. This infectious disease caused by an RNA virus of the Bornaviridae family affects domestic and wild animals, mainly horses and sheeps. In these species Borna virus has a low morbidity, but subclinical persistent infection is probably common (Summer 1995). The virus is transmitted through salivary, nasal and conjunctival secretions, water and contaminated food. Il virus is highly neurotropic, with constant replication in the CNS and spinal cord; however, it can spread throughout the body in infants or the immunocompromised. The disease is characterized, as in our cases, by a nonsuppurative meningo-encephalomyelitis, with infiltration of mononuclear cells that

affects the gray matter of the CNS. The wide spectrum of susceptible species also includes human beings in which it is still uncertain whether the virus is responsible for the development of some forms of nervous disorders. Recent studies have shown a high prevalence of specific antibodies in the serum and/or CSF of patients with neuropsychiatric disorders (Ludwig et al., 1997; Richt et al., 2001).

Chlamydiosis is caused by an obligate intracellular organism of the genus *Chlamydia* that can infect many birds and mammals, including humans. Transmission can occur by digestive, respiratory, venereal and congenital routes, whereas aborted fetuses from infected animals and animals with subclinical infections cause the pathogen to spread into the environment (Nietfeld, 2001; McCafferty, 1990). *Chlamydiae* are primary causes of pneumonia, polyarthritis, conjunctivitis and encephalitis, and sheep is the species most affected. In the cow the infection usually manifests in a generalized form affecting mainly fetuses (vertical transmission) and calves under 6 months of age (Storz et al., 1971). In bibliography have been described cases of sporadic encephalitis in adult cattle with aetiology unclear; often *Chlamydia* infection and viruses has been reported as cause of this disease. (Theil et al., 1998). In our series a chlamydial infection was detected in only one case, a 7-year-old cow with less severe neuropathological lesions than those described previously (Piercy et al., 1999; Storz et al., 1971); probably this animal could have been at an early stage of disease.

Some authors have reported marked seropositivity in people in close contact with animals infected by *Chlamydia*, confirming the hypothesis that animals can be a source of infection for humans (Schachter et al., 1973; Storz et al., 1971). In humans the disease can appear with unapparent forms or with pneumonia, septicaemia and death; sometimes therapy-resistant conjunctivitis can occur. Because of the pathogen's wide diffusion and the few cases reported in humans, many others may not have been recognized (Shewen, 1980). However, cases have been reported of human chlamydiosis transmitted from sheep (Roberts et al., 1967), cattle (Sarateanu et al., 1961) and cats (Schachter et al., 1969; Ostler et al., 1969), or due to laboratory accidents while handling infected samples (Barwell, 1955; Vulgar et al., 1974).

Leishmaniasis is a parasitic disease caused in the Mediterranean by *L. infantum*, a protozoan of the genus *Leishmania*, an obligate intracellular parasite of macrophages and dendritic cells of the skin. It is transmitted by the bite of bloodsucking vectors of the genus *Phlebotomus*; it affects several species, particularly dogs and humans. The disease can be subacute and follow a chronic course with variable symptoms; in more severe cases the animal often dies from renal failure. Other affected organs are those of the reticuloendothelial system (lymph nodes, spleen, liver and bone marrow) and sometimes associated with polymyositis and arthrosynovitis (Matassa, 2007). The CNS is a rather unusual target of the parasite, but some cases have been reported in both dogs and humans (Vinuelas et al., 2001; Melo et al., 2009). In humans the disease is always severe, especially in the immunocompromised, such as HIV-seropositive individuals or transplant recipients. Sometimes, the symptoms are very severe with loss of sensation in the lower limbs, deafness, multiple paralysis of cranial nerve and axonal demyelination and degeneration (Hashim et al., 1995). In recent decades, various climatic factors have changed the normal areal of carriers and new leishmaniasis outbreaks have occurred in areas that were previously considered free, like the foothills of the Alps. Since 1995, outbreaks have been reported in Liguria, Tuscany, Lombardy, Piedmont and Valle D'Aosta, and rarely also in Veneto, Trentino and Friuli (Matassa, 2007).

Nocardiosis is a bacterial disease caused by the Gram-positive bacterium *Nocardia asteroides*. Usually, the infection arises in the lung following inhalation of spores; CNS involvement

occurs in 13% of cases (Bosnic et al., 2010; Cianfoni et al., 2010). As highlighted in our case, *Nocardia* generally causes solitary brain lesions, but cases have also been reported of multiple abscesses, which are very rare in dogs (Smith et al., 2007; Munana, 1996; Kaplan, 1985). The disease has been reported in the brain of immunocompromised dogs and humans due to concomitant diseases (systemic lupus erythematosus or AIDS) or immunosuppressive therapy (cyclosporine, chemotherapy) (Smith et al., 2007). Brain infections can occur after penetrating trauma, due to extension of infection to adjacent structures (internal ear and dental root) or via the bloodstream (Dow et al., 1988; Munana, 1996). Drug therapy is not always effective.

Protothecosis is caused by achlorophil algae of the genus *Prototheca*, including five species widely distributed in the environment, especially in organic matter. It is considered an emerging disease in humans and animals. Infection occurs primarily through skin lesions in contact with contaminated water, but the algae can colonize the skin, even the nails, the respiratory and digestive systems. In cattle the algae cause clinical and subclinical mastitis refractory to treatment, with high economic losses and potential risk to public health; systemic forms are rare (Marques et al., 2006). In dogs and cats it can present both as localized cutaneous and systemic forms (Ginel et al., 1997; Dillberger et al., 1988; Krohne, 2000); respiratory and cutaneous forms have also been reported in the goat (Macedo et al., 2008).

Systemic protothecosis involving the eye and the CNS have been reported in dogs (Stenner et al., 2007); in our case only cerebral lesions were found. In humans the disease has low pathogenicity in immunocompetent subjects, with local indolent skin lesions, whereas in the immunocompromised it may occur as a scattered form and not infrequently associated with such other pathogens as *Candida*, *Staphylococcus aureus*, herpes simplex, and *Cryptococcus* spp. (Lass-Flörl et al., 2007).

Rickettsiosis was diagnosed in only one subject in this series; the disease is caused by an obligate intracellular organism considered to be an intermediate form between a virus and a bacterium of the genus *Rickettsiae*. The disease is a tick-borne zoonosis. Many *Rickettsiae* cause diseases involving the CNS in animals and humans (Parola et al., 2005), one of the most important is *R. conorii* transmitted by the dog tick (*Rhipicephalus sanguineus*) that causes Mediterranean spotted fever in humans. In recent years, the number of cases of rickettsiosis in dogs and humans has increased significantly in Italy, in conjunction with climate change (temperature and rainfall) and environmental (human-dog contact) factors that allow for prolonged survival of the carrier. The most affected regions are Sicily, Sardinia, Latium, Liguria and Piedmont (Garavelli et al., 1990; Cocco et al., 2003; Cascio et al., 2006; Beninati et al., 2002).

Given the wide diffusion of the vector and the pathogen in Italy, the incidence of rickettsiosis was surprisingly low in this series, which may have been due to the fact that many cases go undiagnosed or are asymptomatic without showing neurological signs and are not recognized. Vector control is an indispensable means to prevent diseases transmitted by ticks; rickettsioses should therefore always be included in the differential diagnosis of cases with neurological signs, especially following an arthropod bite.

5. Conclusions

In our investigation, among the many neurological cases potentially attributable to infectious agents, about 27% were confirmed as a zoonosis, but there remain many cases of

inflammation not classified and potentially constituting a health risk. The results of this research show that animals can be an important reservoir for agents of neurozoonoses; therefore, monitoring of neurological diseases of animals should be designed to reduce the number of infections in humans and may be a good model for the study of therapeutic strategies in cases of infection. Neurozoonosis monitoring in animals may also allow for the surveillance of possible emerging or re-emerging infectious agents.

From available literature data it is clear that the population segments most affected by zoonoses are children, the elderly and the immunocompromised, particularly those with AIDS and chemotherapy or transplant recipients. The best way to protect these at-risk groups is through prevention, which can only be done through a national surveillance network. Further studies should also aim to identify the pathogenesis and the possible etiologic agents of idiopathic inflammatory disease or those of "unknown aetiology" to exclude the likelihood of potentially zoonotic agents.

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