Prevalence of antibodies to tickborne encephalitis and West Nile flaviviruses and clinical signs of tickborne encephalitis in dogs in the Czech Republic

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Prevalence of antibodies to tickborne encephalitis and West Nile flaviviruses and the clinical signs of tickborne encephalitis in dogs in the Czech Republic

J. KLIMEŠ, Z. JUŘICOVÁ, I. LITERÁK, P. SCHÁNILEC, E. TRACHTA E SILVA

Blood sera from 151 dogs from areas of the Czech Republic endemic for human tickborne encephalitis (TBE) were examined for the presence of antibodies to TBE and West Nile (WN) flaviviruses by the haemagglutination-inhibition test. Antibodies to TBE virus at titres equal to or exceeding 40 were found in five dogs. Antibodies to WN virus were detected in only one dog that also had a high antibody titre to TBE, suggesting this was a cross-reaction between the two closely related viruses. Three of the dogs (all rottweilers) with a TBE titre of 320 had clinical signs of meningoencephalitis or encephalitis. They all survived after treatment for the clinical signs. It was proved by seroconversion that the disease was caused by the TBE virus in one of these three dogs, and it seems very likely that the virus was responsible for the disease in the other two.

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J. Klimeš, MVDr, CSc,
I. Literák, Doc, MVDr, CSc,
P. Schánilec, MVDr,
Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences, Palackého 1-3, 61242 Brno, Czech Republic
Z. Juřicová, RNDr, CSc,
Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, Valtice, Czech Republic
E. Trachta e Silva, MV,
Private Veterinary Clinic, Batayporã, Mato Grosso do Sul, Brazil

THE human infection caused by the European subtype of tickborne encephalitis (TBE) virus occurs in many endemic areas of continental Europe. The Czech Republic is one of the regions with a high morbidity rate. Between 1955 and 1995 the annual incidence of TBE in people in the former Czechoslovakia was 4·2 (1·4 to 9·9) cases per 100,000 (Hubálek and Halouzka 1996). The virus causes meningoencephalitis with a potentially fatal outcome (Kunz 1992).

In endemic areas dogs come into contact with *Ixodes ricinus* ticks, which are the principal vector of TBE virus, more frequently than people, and as a result a higher seroprevalence has been observed (Matile and others 1979). However, dogs seem to be much more resistant to the clinical disease than people, because TBE has rarely been reported in dogs in Europe (Grešíková and others 1972a, MacKenzie and others 1973, Matile and others 1979, Tipold and others 1993, Weissenböck and Holzmann 1997, Reiner and Fischer 1998) and attempts to produce the clinical disease by experimental inoculation have failed (Grešíková and others 1972b, Elečková and others 1976). Sixl and others (1973) considered the dog as an indicator species for TBE. Similarly, Chiba (1996) monitored dogs in a natural focus of Russian spring-summer encephalitis (RSSE) in Japan. The RSSE virus (an eastern subtype of TBE flavivirus) is antigenically very closely related to TBE virus and is indistinguishable by conventional tests (Hubálek and Halouzka 1996).

The aim of this study was to determine the seroprevalence to TBE virus in dogs living in the city of Brno, its surroundings, and some other areas of endemic human TBE in the Czech Republic. In dogs with antibodies to TBE virus, the possibility of a clinical form of TBE was analysed retrospectively. In addition, antibodies against the West Nile (WN) virus were also studied. This virus is predominantly an endemic avian flavivirus transmitted by mosquitoes, which has also been found in the Czech Republic (Hubálek and others 1989) and which can cause clinical disease in people (Grešíková and Nosek 1981, Hubálek and Halouzka 1996) and dogs (Blackburn and others 1989). There is little information on the prevalence of WN antibodies in dogs (Sixl and others 1973).

MATERIALS AND METHODS

Between May 1997 and October 1998, blood sera were collected from 151 dogs from Brno, other parts of Moravia, and southern, eastern and central Bohemia. The dogs ranged in age from two months to 16 years, and only 10 were under one year of age. There were 94 females and 57 males. There were 19 cross breeds and 47 different pure breeds, with German shepherd dogs (n=22), dachshunds (15), poodles (eight) and rottweilers (eight) being the breeds most represented. The

dogs had a wide range of clinical conditions. Several dogs were receiving health checks being followed up after the successful treatment of an illness. Twenty-seven of the dogs had a neurological disease (epilepsy, meningoencephalitis, vestibular syndrome, commotio cerebri, polyneuropathy, etc).

The blood samples were taken either from an antebranchial vein or from a jugular vein. Serum was separated by centrifugation within two hours and stored at –22°C until analysis. All the dogs with clinical signs of disease were sampled within four days of being first examined.

The sera were examined for antibodies to TBE flavivirus (central European encephalitis serogroup) and WN flavivirus by the haemagglutination-inhibition test (HIT) described by Juřicová and others (1998). The TBE antigen was a commercially available 'HA TBE Antigen' (Imuna). For the preparation of WN antigen, the strain WN EgAr 101 SM12 (Hubálek and others 1989) was used according to the method described by Clarke and Casals (1958). Four units of haemagglutinin were used in all the tests. Titres of 40 and higher were taken as positive (Lennette and Schmidt 1969).

Three dogs with the highest titres were re-examined serologically for TBE antibodies in February and March 1999, 18 to 21 months after the initial examination. One dog with signs of neurological disease was re-tested four days after the initial sampling.

Dogs with a titre equal to or exceeding 320 and with signs of neurological disease are described in greater detail; they were examined by standard clinical, neurological and laboratory procedures and techniques. Cerebrospinal fluid (CSF) was collected from the cerebellomedullary cistern. Antibodies to *Toxoplasma gondii* were tested by indirect fluorescence antibody test (IFAT), using a commercial antigen (Sevatest Toxoplasma antigen IFR; Sevac) and the conjugate Dog IgG – FITC (Sigma); the sera were tested in two-fold dilutions, starting at the titre 40; titres of ≥40 were considered positive. Antibodies to *Neospora caninum* were tested by IFAT, using tachyzoites of the NC–1 strain as the antigen; the sera were tested in two-fold dilutions starting at the titre 80; tires of ≥80 were considered positive. Antibodies to *Borrelia burgdorferi* were tested by ELISA, using a diagnostic kit (Dog EIA Borrelia IgG/IgM; TEST-Line) with *B burgdorferi* s. 1. (*B afzelii*, strain KC90CZ). The result was evaluated in terms of the index of positivity, according to the manufacturer's instructions.

RESULTS

Antibodies to TBE were found in five of the 151 dogs (3·3 per cent), one with a titre of 40, three with a titre of 320 and one with a titre of 1280. This last dog also had antibodies against WN virus, with a titre of 80, and it was the only dog with detectable antibodies to this virus (Table 1).

All the dogs which were serologically positive to TBE virus had been correctly vaccinated against rabies and distemper, and ticks were repeatedly observed on them by their owners several days to weeks before they were examined and sampled.

Dog 1 This dog was examined in August 1997 after becoming progressively disoriented for five days, aggressive toward its owner when being handled, and finally unable to walk. Its rectal temperature was within the normal range. A neurological examination revealed disorientation, aggressiveness, neck stiffness, generalised ataxia and tetraparesis. Passive manipulation elicited signs of pain, and its spinal reflexes were normal or slightly increased. The findings were consistent with a diagnosis of meningoencephalitis. Therapy consisted of rest, dexamethasone and ampicillin. The dog recovered uneventfully. No antibodies to *T gondii*, *N caninum* or *B burgdorferi* were found.

Dog 2 In September 1997 this dog developed acute gastroenteritis which resolved after symptomatic treatment. No signs of neurological disease were observed by the owner either before or for 18 months afterwards. No tests for antibodies to *T gondii*, *N caninum* and *B burgdorferi* were applied.

Dog 3 This dog was examined in June 1997 after showing signs of nervousness for five days, followed by lethargy, inappetence, ataxia, vomiting, and an unwillingness to move. An examination confirmed these observations. Its body temperature was normal throughout the course of the disease. Seven days after onset of the disease, restlessness, aggressiveness, and myoclonus of the head, neck and front limbs developed. Abnormal neurological findings included stupor, salivation, low head carriage, cervical pain, dysphagia, increased facial sensitivity, markedly increased patellar reflex, and increased tibialis cranialis reflex. The dog's postural reactions were normal.

The results of a complete blood count and serum biochemical analyses were within reference ranges. No antibodies to *T gondii* and *B burgdorferi* were detected, but antibodies to *N caninum* were detected at a titre of 160. A CSF analysis revealed an increased total protein level (0·45 g/litre), and mixed pleocytosis with a leucocyte count of 50 x 10^6/litre. A cytological examination revealed rested monocytes, activated neutrophils and lymphocytes, and an increased proportion of plasma cells. The findings were compatible with a diagnosis of meningoencephalitis localised in the brainstem.

Therapy included rest, dexamethasone, phenobarbital and chlorpromazine, ampicillin and trimethoprim sulpha, fluid therapy with lactated Ringer's solution, and dextrose. The clinical state of the dog improved during nine days in hospital, and two weeks after discharge it was clinically normal. A follow up clinical examination after more than a year was normal.

Dog 4 In June 1997 this dog developed septic peritonitis as a complication of pyometra. The dog was discharged from the hospital after 10 days following substantial clinical improvement. Its fate is unknown. Tests for antibodies to *T gondii*, *N caninum* and *B burgdorferi* were not applied.

Dog 5 This dog was examined in September 1998, one day after it had become inappetent, with pica, vomiting, anxiety, ataxia, falling to the right, hyperaesthesia and generalised tonic seizures. Its diet included raw goat's milk. Stupor, miosis, mus-

TABLE 1: Characteristics of the five dogs seropositive to tickborne encephalitis (TBE) and West Nile (WN) flaviviruses

Dog	Breed	Age (years)	Sex	TBE titre (first sampling/ second sampling)	WN titre	*T gondii* titre	*N caninum* titre	*B burgdorferi* titre	Neurological signs
1	Rottweiler	3·5	M	320/40*	0	0	0	0	Meningoencephalitis
2	Brazilian Fila	1	M	1280/320*	80	Not tested	Not tested	Not tested	–
3	Rottweiler	1	M	320/40*	0	0	160	0	Meningoencephalitis
4	Dachshund	9	F	40/not tested	0	Not tested	Not tested	Not tested	–
5	Rottweiler	8	F	80/320†	0	160	0	0	Encephalitis

* Second sampling after 18 to 21 months, † Second sampling after four days
Antibodies to WN, *T gondii*, *N caninum* and *B burgdorferi* were tested during the first sampling. M Male, F Female

cle tremor, and occasional tonic seizures were observed. Later in the course of the disease, clonic spasms of the thoracic limbs and the whole body, opisthotonus, excitation after acoustic stimulation, facial myoclonus, and spinal hyperreflexia developed.

Initially, the dog was anaemic with a packed cell volume of 0·24 litre/litre, and it later developed a more pronounced anaemia, mild leucocytosis (21·2 x 10^9/litre), neutrophilia (17·8 x 10^9/litre), and a left shift – bands (1·48 x 10^9/litre) were detected. Biochemical tests revealed prerenal azotaemia with high concentrations of creatinine (278·8 µmol/litre) and blood urea nitrogen (15·9 mmol/litre). Antibodies to *T gondii* were detected at a titre of 160. No antibodies to *B burgdorferi* or *N caninum* were detected. Immunological tests revealed a high lysozyme concentration, a high activity in the chemiluminescence test, and increased phagocytic activity of leucocytes, consistent with an inflammatory process. The inhibition of the lymphocyte transformation test suggests that the dog's immunity may have been compromised.

An evaluation of the CSF, after correction for artificial bleeding (Wilson and Stevens 1977), indicated a high total protein concentration (2·09 g/litre), a high total leucocyte count (200 x 10^6/litre) and a high mononuclear cell count (31·2 x 10^6/litre). These findings correspond with the diagnosis of encephalitis.

Therapy included rest, dexamethasone, diazepam, enrofloxacin, and fluid therapy, as in the previous case. After four days, the dog's seizures ceased, after six days, it began to drink and eat, and after 12 days, it was discharged from the hospital. After six months, the generalised ataxia persisted.

DISCUSSION

The prevalence of dogs with TBE antibodies (3·3 per cent) was similar to the prevalence reported by Matile and others (1979), who found antibodies to TBE virus in 3·6 per cent of 657 dogs examined in endemic areas of Switzerland. Chiba (1996) recorded antibodies against RSSE (an Eastern subtype of TBE) in eight of nine dogs (88·9 per cent) in an area of Hokkaido with a suspected human case of RSSE, but in only 1·3 per cent of 150 dogs from other parts of Hokkaido examined by neutralisation test. Antibodies to TBE were detected by HI test in eight (22 per cent) of 36 dogs from endemic areas of Austria (Sixl and others 1973), but when only titres of 40 and higher were considered as positive, only three dogs (8·3 per cent) were positive.

The seroprevalence in the dogs from the Czech Republic was similar to the prevalence of TBE antibodies in people (2 to 38 per cent) in various endemic areas of former Czechoslovakia (Blaškovič 1970). The prevalence in people in Switzerland reported by Matile and others (1979) was lower (0·5 per cent) than in dogs (3·6 per cent).

The samples were collected throughout the year, but positive titres and possible clinical disease were observed only from June to September, coinciding with the increase in the tick population (Kunz 1992).

Sixl and others (1973) found antibodies to WN virus in 33·3 per cent of 36 dogs, but when only tires above 40 were considered as positive, the prevalence was only 13·9 per cent. The identical or higher HI titres against WN in dogs which were seropositive to TBE were interpreted by these authors as cross-reactions between closely related viruses. In the present study, the prevalence of WN antibodies was much lower (0·7 per cent), probably in association with the lower prevalence of TBE antibodies (3·3 per cent versus 8·3 per cent). As in the study by Sixl and others (1973), WN antibodies were found only in a dog with a high titre against TBE. Although WN virus may also cause clinical disease in dogs (Blackburn and others 1989), the titre against WN virus was probably caused by a cross-reaction, because in a HI test, the TBE virus cross-reacts with other viruses within the B group of former arboviruses, where the WN virus also belongs (Clarke and Casals 1958, Theiler and Downs 1973).

In central Europe, clinical TBE infection has been conclusively demonstrated in about 15 dogs by means of immunohistology, immunocytochemistry or repeated serological examinations of serum and CSF, using several techniques simultaneously (Tipold and others 1993, Weissenböck and Holzmann 1997, Reiner and Fischer 1998). They were all large breed dogs from Switzerland, Austria or Germany. They usually had a high body temperature and an acute onset of rapidly progressive neurological signs. The most frequently reported neurological signs were abnormal behaviour, ataxia, limb paresis, myoclonus, seizures, miosis, and cranial nerve deficits. A multifocal distribution with forebrain, brainstem and cervical spinal cord signs was characteristic. Most of the dogs had leucopenia and lymphopenia. The dogs' CSF had a high protein concentration and mononuclear pleocytosis (Tipold and other 1993, Reiner and Fischer 1998). Only two of the dogs survived after intensive therapy, one with persistent, severe lower motor neuron paresis of its thoracic limbs (Reiner and Fischer 1998). The other dogs died or had to be euthanased. A closely related louping ill virus, endemic to the British Isles and Norway, causing primarily meningoencephalomyelitis in sheep, was demonstrated by virus isolation in two border collies. They both died with severe neurological signs of ataxia, neck stiffness, excitability, paddling, nystagmus and opisthotonus, five weeks after whelping (MacKenzie and others 1973).

In this study, the three seropositive rottweilers showed clinical signs of acute meningoencephalitis or encephalitis that were probably caused by TBE virus. However, this conclusion is not certain for two of them, because samples of serum taken several weeks apart were not examined, and their CSF was not examined for TBE antibodies. The persistence of lower titres after 18 to 21 months is also suggestive of a previous acute infection. The clinical signs in these dogs resembled those described in proven cases of TBE. The increased protein concentration and pleocytosis in the CSF are also in agreement with the findings reported by Tipold and others (1993) and Reiner and Fischer (1998). Mononuclear pleocytosis, characteristic of viral encephalitis, was observed in dog 5, but the CSF was evaluated in only two of the dogs. The major differences were the absence of fever and leucopenia, and the relatively rapid recovery.

Generalised ataxia persisted only in dog 5; a similar result was described in a rottweiler by Reiner and Fischer (1998). The marked seroconversion of antibodies to TBE observed in dog 5 was associated with the acute clinical stage of disease. The authors therefore consider that this eight-year-old female rottweiler suffered from acute clinical TBE.

The most important differential diagnoses of TBE in dogs are rabies and distemper. The possibility of rabies can be discounted because the three dogs had been vaccinated correctly, and they all recovered rapidly. A similar argument applies to a lesser extent to distemper.

The possibility of bacterial encephalitis cannot be completely excluded, because the CSF was not cultured. Nevertheless, neither phagocytosis of bacteria nor free bacteria were observed in cytological preparations of CSF.

The over-representation of rottweilers in this survey is noteworthy, since about 30 per cent of dogs with proven TBE have been rottweilers (Tipold and others 1993, Reiner and Fischer 1998).

The presumable source of the TBE infection was the tick *I ricinus*. It is remarkable from the epidemiological point of view that dog 5 was regularly fed raw goat's milk. This is a very unusual feeding practice in the Czech Republic, and it is possible that the TBE virus was transmitted via the milk of a viraemic goat, which is a well-documented mode of transmission in some outbreaks of human TBE in central Europe (Grešíková and others 1975).

The high titres of antibodies to TBE are not always associated with neurological signs. The highest antibody titre (1280) and follow up titres (320) were both recorded in dog 2, which had no clinical signs of neurological disease either before or after the initial examination. Nevertheless, in people, TBE usually has a biphasic course. In the initial phase, lasting one to eight days, the non-specific signs may include vomiting and diarrhoea; this is followed by an asymptomatic period after which 20 to 30 per cent of patients develop neurological symptoms, or the disease resolves without entering the second stage (Kunz 1992). In dog 2, the signs of 'gastroenteritis' may represent the initial stage (so far undescribed in dogs), which did not progress to the stage with neurological abnormalities.

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SHORT COMMUNICATIONS

Pregnancy following in vitro fertilisation of canine oocytes

G. C. W. ENGLAND, J. P. VERSTEGEN, D. A. HEWITT

TO date, there have been no reports of the successful transfer of in vitro matured canine oocytes. Work by Mahi and Yanagimachi (1976), Hewitt (1997) and Shimazu and Naito (1996) showed that dog spermatozoa could penetrate immature homologous oocytes, and recently Fulton and others (1998) fertilised bitch oocytes by intracytoplasmic sperm injection. In the fox, embryos have been produced in vitro, and one subsequently survived to the morula stage (Farstad and others 1993a, b). Similar studies in the dog have shown that the highest rate of embryo production occurred following the in vitro fertilisation of ovulated oocytes recovered from the ovarian bursa or oviduct (Metcalfe 1999), and although a surgical method for recovering bitch embryos has been described (Archbald and others 1980), the technology has not been successfully used for embryo transfer. Work in the authors' laboratory has examined the in vitro maturation of oocytes and has demonstrated that the selection of oocytes which have acquired meiotic competence through adequate intrafollicular growth, is important for in vitro maturation (Hewitt and England 1998b). In this short communication, the collection of oocytes from bitches in oestrus, their in vitro fertilisation and subsequent transfer are described.

Four anoestrous beagle bitches, aged three to eight years and housed in pairs, were treated daily with 5·0 µg/kg cabergoline (Galastop; Vetem) for between 12 and 15 days until the onset of vulval bleeding (Verstegen and others 1999). Subsequently, the bitches were examined daily with vaginal cytology and measurement of peripheral plasma progesterone concentrations (Eckersall and Harvey 1987). All bitches

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G. C. W. England, BVetMed, PhD, DVetMed, FRCVS, DVR, DipVRep, DipECAR, DipACT, **D. A. Hewitt,** BSc, PhD, Royal Veterinary College, University of London, Hawkshead Lane, North Mymms, Hatfield, Hertfordshire AL9 7TA **J. P. Verstegen,** DVM, MSc, PhD, DipECAR, Faculté de Médecine Vétérinaire, Université de Liège, Boulevard de Colonster, Liège, Belgium

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J. Klimes, I. Literák, P. Schánilec, Z. Juricová and E. Trachta e Silva

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