Testing for Vector-Transmitted Microorganisms in Dogs with Meningitis and Meningoencephalitis of Unknown Aetiology

Kali Lazzerini1*, Andrea Tipold2, Marion Kornberg3, Cornelia Silaghi4, Andreas Mietze5, Antina Lübke-Becker6, Anneliese Balling7, Martin Pfeffer5, Lothar H. Wieler6, Kurt Pfister4 and Barbara Kohn1

1Clinic of Small Animals, Freie Universität Berlin, Germany
2Department of Small Animal Medicine and Surgery, University of Veterinary Medicine, Germany
3Small Animal Clinic, Trier, Germany
4Institute of Comparative Tropical Medicine and Parasitology, LM-University, Munich, Germany
5Institute for Microbiology, University of Veterinary Medicine, Germany
6Institute of Microbiology and Epizootics, Freie Universität Berlin, Germany
7Institute of Animal Hygiene and Veterinary Public Health, University of Leipzig, Germany

Abstract

In most cases of inflammatory central nervous system (CNS) diseases in dogs, infectious agents remain undetected. Immunopathological studies suggest that such antigens may trigger an autoimmune response (“hit-and-Run hypothesis”) in some patients. In order to define the role of vector-borne pathogens in the aetiology of steroid-responsive meningitis-arteritis (SRMA) or meningoencephalomyelitis of unknown aetiology (MUE), blood and cerebrospinal fluid (CSF) of dogs were analysed for such pathogens.

66 client-owned dogs were included in the prospective multicenter study over a two year period. They were classified into 3 groups: 1] trauma group: dogs with non-inflammatory CNS diseases (n=21), 2] dogs with MUE (n=22), 3] dogs with SRMA (n=23).

DNA of *A. phagocytophilum* was found in EDTA-blood of 4 dogs [SRMA group]. Serological and PCR analyses for *E. canis* were negative in blood and serum of all dogs. *B. henselae* DNA was detected in blood of 1 dog [SRMA group]. There were no significant differences between the 3 groups regarding the seroprevalence of *Bartonella* spp. [n=61] and *B. burgdorferi* sensu lato [n=61]. Neither antibodies against TBEV in serum nor DNA of vector-transmitted agents was found in CSF in any of the dogs. Pasteurellaceae spp. DNA was detected in 3 dogs of the trauma group, suggesting contamination.

There was no correlation between the presence of *E. canis* or *B. henselae* DNA or elevated antibody titers against *E. canis, Bartonella spp., TBEV or B. burgdorferi sensu lato and inflammatory CNS diseases. *A. phagocytophilum* may play a role as a trigger of a secondary immunopathy.

ABBREVIATIONS

CNS: central nervous system; SRMA: steroid-responsive meningitis-arteritis; MUE: meningoencephalitis of unknown etiology; CSF: cerebrospinal fluid; PCR: polymerase chain reaction; DNA: deoxyribonucleic acid; TBEV: tick-borne encephalitis virus; IFAT: immunofluorescence antibody test; ELISA: enzyme linked immunosorbent assay; MRI: magnetic resonance imaging; CT: computed tomography; EDTA: ethylenediaminetetraacetic acid; rRNA: ribosomal ribonucleic acid; NME: necrotizing meningoencephalitis; GME: granulomatous meningoencephalitis

INTRODUCTION

Inflammatory lesions of the central nervous system [CNS] in dogs remain challenging for clinicians. Diseases causing CNS inflammation without a definitive diagnosis are a heterogeneous group currently known as meningoencephalitis of unknown aetiology [MUE] [1]. The pathogenesis of these diseases is still unknown, but a multifactorial aetiology with infectious, genetic, and immunopathological components may be responsible [2-4]. Steroid-responsive meningitis-arteritis [SRMA] is another inflammatory CNS disease of unknown pathogenesis. It is...
considered an immune-mediated disease, although research to
determine its aetiology is ongoing [5].

Vector-borne diseases, i.e. diseases caused by pathogens
that are transmitted to vertebrate hosts by ectoparasitic
vectors, are amongst the suspects to either directly or indirectly
induce inflammatory diseases of the CNS [2,4,6]. The vector-
borne pathogens *Anaplasma phagocytophilum*, *Ehrlichia canis*,
*Bartonella* spp., *Borrelia burgdorferi* sensu lato, and tick-borne
encephalitis virus [TBEV] have all been suspected to cause
neurological signs in dogs [7-11].

*A. phagocytophilum* is the pathogen responsible for
granulocytic anaplasmosis. Single cases of dogs with neurological
signs have been PCR-positive for *A. phagocytophilum* in blood
[12]. Other studies, however, have failed to confirm a correlation
between elevated antibody titres against *A. phagocytophilum*
and neurological deficits [13] or to detect the DNA of *A.
phagocytophilum* in the blood or CSF of dogs with neurological
signs [7].

Neurological signs such as ataxia and paraparesis have been
described in the chronic form of canine monocytic ehrlichiosis,
which is caused by *E. canis* [8]. A correlation between infection
with *E. canis* and neurological signs remains unclear. Infections
with *Bartonella* spp. are often subclinical but can lead to the
development of clinical signs of canine bartonellosis. The most important species in dogs are *Bartonella henselae* [cat
scratch disease] and *B. vinsonii* ssp. *berkhoffii* [14]. A few case
reports have described dogs infected with *Bartonella* spp.
that developed neurological signs of meningoaraculuneuritis
[15] or meningoencephalitis [16,17]. The DNA of *B. vinsonii*
ssp. *berkhoffii* was detected in the brain of one dog with
granulomatous meningoencephalitis [GME] in a study of 109
dogs with neurological diseases [9].

Infection with *B. burgdorferi* sensu lato can also cause
neurological signs in humans. To our knowledge, a correlation
between infection with *B. burgdorferi* sensu lato [or any other
*Borrelia* spp.] and encephalomyelitis in dogs has not yet been
documented.

Tick-borne encephalitis [TBE] in Central Europe is caused by
a flavivirus. If clinical signs appear, the disease generally has a
fatal outcome within 4-7 days [11,18].

We analysed the blood and CSF of dogs to investigate the role
of vector-borne microorganisms in the pathogenesis of SRMA
and MUE.

**MATERIALS AND METHODS**

**Animals**

We recruited 66 client-owned dogs between 12/2009
and 11/2011 presented to the Small Animal Clinic, Freie
Universität Berlin; the Department of Small Animal Medicine
and Surgery, University of Veterinary Medicine, Hannover;
and the Small Animal Clinic in Trier. The patients were classified
into three groups: trauma, MUE, and SRMA. The trauma group
included 21 dogs with non-inflammatory CNS diseases [eg.
intervertebral disc disease], the MUE group included 22 dogs
with meningoencephalitis, and the SRMA group included 23
dogs with SRMA. The number of patients was determined by
animals that could be gathered in a period of two years with the
established disease profiles and available samples.

The criteria for inclusion were a diagnostic work-up, including
CSF analysis and diagnostic imaging [magnetic resonance
imaging [MRI] or computed tomography [CT] with intravenous
contrast medium], and sufficient CSF, EDTA-treated blood, and
serum available for further analysis. A presumptive diagnosis of
MUE was declared when a dog showed neurological deficits,
pleocytosis [>3 cells/µL] and elevated protein concentrations in
the CSF, multifocal lesions with contrast enhancement on MRI
or CT scans or no signs of neoplasia on the CT scan [4]. SRMA
was diagnosed if the signalment, clinical, and neurological signs
were consistent with the disease and if the CSF analysis showed
polymorphonuclear pleocytosis [5]. Dogs with a low total
nucleated-cell count were excluded unless they had a history of
corticosteroid treatment before CSF collection [19-21]. Treatment
with corticosteroids before CSF collection was documented for
all cases (see Table 1). Five dogs of the MUE group, three dogs of
the SRMA group, and 12 dogs of the trauma group had received
corticosteroids prior to diagnostic testing (Table 1). Antibiotic
treatment before CSF collection was documented for all cases
(Table 1).

The number of dogs that originated from or had travelled to
various geographical areas was documented (Table 2). Owner
permissions were obtained for the use of the samples, and the
study adhered to university guidelines.

**DNA extraction and PCR analysis**

PCR was performed on blood and CSF samples to detect
the DNA of *A. phagocytophilum*, *E. canis*, and *Bartonella* spp.
A qualitative eubacterial PCR was performed on CSF samples. Only
dogs that originated from or had travelled to a foreign country
where *E. canis* was endemic [Southern and Eastern Europe] and
dogs with an unknown travel history were tested for *E. canis*
[n=28].

**A. phagocytophilum and E. canis**

DNA extraction was performed using the High Pure PCR
Template Preparation Kit [Roche Applied Science, Mannheim].
Real-time PCR was performed with HotStarTaq according to the
manufacturer’s instructions [Applied Biosystems, Carlsbad] as
previously described [22-24]. The target genes were msp2 for *A.
phagocytophilum* and p30-10 for *E. canis*.

**Table 1:** Medication received prior to CSF collection for the dogs in the
three groups MUE, SRMA, and trauma.

<table>
<thead>
<tr>
<th>Group</th>
<th>Antibiotics [number of dogs]</th>
<th>Corticosteroids [number of dogs]</th>
<th>Non-steroidal anti-inflammatory drugs [number of dogs]</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUE [22]</td>
<td>12</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>SRMA [23]</td>
<td>14</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>Trauma [21]</td>
<td>1</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>20</td>
<td>25</td>
</tr>
</tbody>
</table>

Abbreviations: MUE: meningoencephalitis of unknown aetiology; SRMA: steroid-responsive meningitis-arthritis.
Central as previously described [27].

with a quantitative ELISA for the C6 peptide [IDEXX Laboratories, Borrelia antibody titres ≥1:128 in the IFAT were further analysed. The ELISA for antibodies against E. canis, A. phagocytophilum, and Bartonella spp. was performed as previously described [25]. The species were identified by restriction analysis of the PCR products [25].

**Qualitative eubacterial PCR**

A qualitative eubacterial PCR was performed on the CSF samples of 62/66 dogs to exclude the presence of other bacteria in the CSF. PCR targeting the highly conserved 16S rRNA gene was performed as previously described [26]. Amplicons corresponding to the 331-797 base-pair region of the *Escherichia coli* 16S RNA gene were sequenced [single-stranded] by LGC Genomics GmbH, Berlin and were compared to nucleotide sequences from relevant databases [NCBI/BLAST].

**Serological testing**

Serological testing was performed to detect antibodies against TBEV [ELISA], *E. canis* [IFAT], *B. burgdorferi* sensu lato [IFAT, or *C. felis* ELISA if the IFAT antibody titre ≥ 1:128], and *Bartonella* spp. [ELISA]. Serum antibodies against TBEV were tested using the IgG All Species ELISA kit [Progen Biotechnik GmbH, Heidelberg]. The IFATs for *E. canis* and *B. burgdorferi* sensu lato [MegaScreen FLUO EHRlichia canis and MegaScreen FLUO BORRELLA canis test kits, MegaCor Diagnostik GmbH, Hörbranz] were performed following the manufacturer’s instructions. Titres of 1:64 or higher for *E. canis* were considered positive. The *Borrelia* IFAT is a qualitative method for detecting antibodies against *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*. Sera with anti-*Borrelia* antibody titres ≥1:128 in the IFAT were further analysed with a quantitative ELISA for the C6 peptide [IDEXX Laboratories, Ludwigsburg]. Titres below 1:128 were interpreted as negative. The ELISA for antibodies against *Bartonella* spp. was performed as previously described [27].

**Statistical analysis**

Groups were compared with non-parametric tests [Kruskal-Wallis and Fisher’s exact tests] [SPSS 17.0 for Windows, SPSS Inc., USA]. A P-value <0.05 was considered significant.

RESULTS AND DISCUSSION

PCR test results [CSF and blood]

PCR testing of the CSFs did not detect any amplification products specific for the vector-borne pathogens *A. phagocytophilum*, *E. canis*, or *Bartonella* spp. DNA of *A. phagocytophilum* was detected in the EDTA-blood of four dogs [all in the SRMA group, 17.4%]. The difference between the three groups was statistically significant [p=0.012]. No differences were detected amongst dogs from different regions of Germany [Berlin, Trier, or Hannover] or between dogs that had stayed in Germany and dogs that had travelled to Southern or Eastern Europe. The PCR analyses of blood for *E. canis* was negative for all dogs [n=28]. *B. henselae* DNA was detected in the blood of one dog [SRMA group], and *Pasteurellaceae* spp. DNA was detected in three dogs of the trauma group.

Serological results

None of the dogs had detectable antibody titres against TBEV or *E. canis* in the sera.

Fourteen of 61 dogs had *B. burgdorferi* sensu lato serum antibody titres ≥1:128 using IFAT [seroprevalence 22.9%]. The seroprevalences of *B. burgdorferi* sensu lato did not differ significantly amongst the three groups [MUE, SRMA, and trauma]. Sera of the 14 IFAT-positive dogs were tested for the C6 peptide using ELISA; the antibody titre was >10 U/mL in two dogs of the MUE group and in one dog of the SRMA group. The titre levels established with the IFAT did not correlate with the C6 titres.

The antibody titre against *Bartonella* spp. in sera was measured in 61 dogs using ELISA. The seroprevalence averaged 83.6% The three groups did not differ significantly amongst dogs from different regions of Germany [Berlin, Trier, and Hannover] or between dogs that had stayed in Germany and those that had travelled to Southern or Eastern Europe.

DISCUSSION

The blood and CSFs of dogs with SRMA and MUE were analysed for vector-transmitted microorganisms. The results were negative in the majority of cases. The negative PCR analyses in CSF corroborated the results of a previous study [Barber et al., 2010] that tested the CSFs from 74 dogs with neurological symptoms. DNA of *E. canis*, *A. phagocytophilum*, spotted fever group *Rickettsia, Bartonella* spp., and *Borrelia* spp. was not detected in these samples.

*A. phagocytophilum* DNA was not detected by PCR in the blood and CSF of dogs with neurological deficits in another study [7]. The DNA of *A. phagocytophilum* in the CSF, however, may have remained undetected due to a bacterial load in the brain below the limit of detection or to suboptimal PCR conditions.

In our study, DNA of *Bartonella* spp. was not detected in CSF, which can be due to a low bacterial load in dogs [28,29]. Brain tissues from 35 dogs were evaluated by PCR, and the DNA of *B.
**henselae** was detected in the brain of only one dog with GME [9]. Future studies should thus employ pre-enrichment culture methods [28,30] for the detection of *Bartonella* spp. in CSF.

PCR testing of blood and CSF for *E. canis* DNA and antibody testing in sera were negative in all dogs tested [29/66 dogs had travelled to Southern, Northern, or Eastern Europe or had an unknown travel history and were therefore tested], corroborating the results of a previous study [9].

Qualitative eubacterial PCR was performed on the CSFs of 62/66 dogs. The DNA of *Pasteurellaceae* sp. was detected in the CSF of three dogs [trauma group]. Species identification was not successful in any of the cases. Most species of *Pasteurellaceae* are well adapted to mucosae, so contamination with oral *Pasteurellaceae* cannot be ruled out. These bacteria are not likely to play a relevant role in CNS inflammation.

PCR analyses for *A. phagocytophilum* DNA in blood were positive in 4/65 patients. All dogs belonged to the SRMA group [prevalence 17.4%]. Further information about these patients is listed in Table 3. Canine anaplasmosis and SRMA have overlapping clinical features [e.g. fever]. None of the dogs showed typical laboratory abnormalities of anaplasmosis [e.g. thrombocytopenia]. These results may imply that infection with *A. phagocytophilum* can play a role in the pathogenesis of immune-mediated CNS inflammation. The hit-and-run hypothesis describes such a phenomenon [31]. When an infectious agent induces a CNS inflammatory response, e.g. by molecular mimicry, the response can lead to clinical signs after the infectious agent has been eliminated or has not even entered the CNS [32]. *A. phagocytophilum* infections can also lead to secondary immune-mediated processes such as immune-mediated thrombocytopenia or anaemia and polyarthritis. Platelet-bound antibodies were detected in humans [Wong and Thomas, 1998] and in dogs with granulocytic anaplasmosis [60-80%] [33,34], and two dogs with granulocytic anaplasmosis had a positive Coombs’ test [35].

The DNA of *B. henselae* was detected in the EDTA-blood of one patient [SRMA group]. In a previous study in Germany using the same method, the prevalence of *B. henselae* in dogs determined by PCR [of blood] was much higher than in published prevalence studies in developed countries [30]. The positive PCR in our study thus does not necessarily imply a causal relationship between infection and the development of neurological signs.

Antibody titres against *Bartonella* spp. were measured in sera using ELISA. Seroprevalences varied between 73.3% [MUE group] and 90.5% [trauma group]. The seroprevalence for the entire cohort was 83.6%, which was much higher than previously reported seroprevalences [36]. In our study, seroreactivity to various *Bartonella* spp. was tested. We do not know if the dogs were asymptomatic carriers of non-pathogenic strains or if the three groups were infected with pathogenic species, but seroprevalence did not differ significantly amongst the three groups.

Fourteen of 61 dogs had IFAT antibody titres against *B. burgdorferi sensu lato* ≥1:128 [seroprevalence 22.9%], with no significant differences amongst the groups. Seropositivity is insufficient to determine causality between infection and development of clinical signs. Serologic testing for the **C**<sub>s</sub> peptide can differentiate between naturally infected and vaccinated dogs. A **C**<sub>s</sub>-ELISA was performed in 13 of the 14 dogs with elevated IFAT titres. Two dogs of the MUE group and one dog of the SRMA group had elevated **C**<sub>s</sub> titres. We cannot exclude an association between the development of CNS inflammation and seropositivity for *B. burgdorferi sensu lato*, although a previous experimental study did not demonstrate that infection with *B. burgdorferi sensu lato* led to inflammatory disease of the CNS [10]. The evaluation of CSF for antibodies might provide more information about clinical relevance [10]. In a study in Sweden, all CSF samples from dogs with neurological symptoms were negative for *A. phagocytophilum* and *B. burgdorferi sensu lato* antibodies and DNA, although many of the dogs had serum antibodies for both agents [7]. In the present study, insufficient CSF was available for further analysis.

Serologic testing for antibodies against TBEV in sera was negative in all patients, which was not unexpected. More than 90% of all notified TBE cases have been in Bavaria, Baden-Württemberg, and Southern Hesse. The three clinics involved in the present study do not receive many patients from these areas. The travel histories of the dogs in our study (see Table 2) and the current known distribution of TBEV in Europe suggest the possibility of finding dogs with a previous infection with TBEV [11,37,38].

The diagnosis of the relevant pathogens could be enhanced by the measurement of paired antibody titres in sera [e.g. for *Bartonella* spp. and *A. phagocytophilum*]. A 4-fold increase in antibody titres is highly suggestive of an active infection. In the present study, however, second serum samples were not available for analysis.

A limitation of this study was that five dogs [one in the MUE group and four in the SRMA group] were pre-treated with doxycycline prior to referral and CSF collection. The pre-treatment with doxycycline in these five cases may have led to false negative PCRs for the pathogens tested [39]. Interestingly, one of these patients was PCR-positive for *A. phagocytophilum*, which could have been due to a low dosage of the medication or to an inappropriate administration by the owner. All antibiotics can potentially cross a blood-brain barrier disrupted by inflammation. Antibiotic treatment with penicillin derivatives [12], fluoroquinolones [11], cefalosporin [1], or clindamycin [1] prior to CSF examination may have led to false negatives in the eubacterial PCR analysis.

In this study, the MUE group included a heterogeneous collection of inflammatory CNS diseases. In similar studies with an emphasis on pathological features, post-mortem examinations were available, and inflammatory disease states were further differentiated. In one study, 75 cases of GME, NME, or MUE were evaluated by PCR for vector-borne agents [from brain tissue or CSF], and the DNA of *B. henselae* was detected in only one dog [9]. A limitation of pathological studies is that examinations are conducted in the late stages of disease when significant tissue inflammation has already occurred and possible infectious triggers may have been eliminated. The current clinically oriented study focused on CNS inflammation rather than exact histological classifications of disease. This focus allowed us to achieve adequate group sizes and to examine living animals.
before the final stage of the disease. Examining the presence of pathogens in an earlier stage of inflammation may have increased the likelihood of their detection, assuming they play a role as triggers of immune responses as stipulated in the hit-and-run hypothesis [40]. These negative results from living and diseased [or dead] animals indicate that extended experimental studies with an emphasis on immunopathological processes are needed to determine the potential immunogenic influence of vector-borne pathogens on CNS inflammation.

CONCLUSION

*E. canis* and TBEV appear to be rare causes of inflammatory CNS disease. Neither the DNA of *E. canis* nor antibodies against either pathogen were detected in dogs with MUE or SRMA from various regions of Germany. *B. burgdorferi* sensu lato is an unlikely cause of inflammatory CNS diseases in dogs. Infection with *Bartonella* spp. was not correlated with and MUE or SRMA. *A. phagocytophilum*, however, may play a role as a trigger of a secondary immunopathy.

ACKNOWLEDGEMENTS

We would like to thank all members of the clinical staff for helping with patient care and treatment, especially Miss Ariana Maiolini for her support.

Conflict of interest

The authors certify that they have no affiliations with or involvement in any organisation or entity with any financial interest [such as honoraria; educational grants; participation in speakers’ bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements] or non-financial interest [such as personal or professional relationships, affiliations, knowledge, or beliefs] in the subject matter or materials discussed in this manuscript.

REFERENCES


