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Role of ducks in the transmission cycle of tick-borne encephalitis virus?

Friederike Michel[1] | Ute Ziegler[1] | Christine Fast[1] | Martin Eiden[1] | Christine Klaus[2] | Gerhard Dobler[3] | Karin Stiasny[4] | Martin H. Groschup[1]

[1]Friedrich-Loeffler-Institut, Institute of Novel and Emerging Infectious Diseases, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany

[2]Friedrich-Loeffler-Institut, Institute of Bacterial Infections and Zoonoses, Federal Research Institute for Animal Health, Jena, Germany

[3]Department of Virology and Rickettsiology, Bundeswehr Institute of Microbiology, Munich, Germany

[4]Center for Virology, Medical University of Vienna, Vienna, Austria

Correspondence
Martin H. Groschup, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Novel and Emerging Infectious Diseases, Greifswald-Insel Riems, Südufer 10, 17493 Greifswald-Insel Riems, Germany.
Email: martin.groschup@fli.de

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Abstract

Tick-borne encephalitis virus (TBEV), a member of the family Flaviviridae, is the most important tick-transmitted arbovirus in Europe. It can cause severe illnesses in humans and in various animal species. The main mechanism for the spread of TBEV into new areas is considered to be the translocation of infected ticks. To find out whether ducks can function as a natural virus reservoir in addition to serving as passive transport vectors, we carried out an experimental TBEV challenge study to reveal their susceptibility and resulting pathogenesis. Nineteen ducks were inoculated subcutaneously with TBEV strain 'Neudoerfl' and monitored for 21 days. Blood, oropharyngeal and cloacal swabs were collected throughout the experiment and organ samples upon necropsy at the end of the study. All samples were tested for TBEV-RNA by real-time polymerase chain reaction. TBEV-specific antibodies were determined by virus neutralization test and ELISA. Organ samples were examined histopathologically and by immunohistochemistry. The inoculated ducks did not show any clinical symptoms. TBEV-specific RNA was detected in all brain samples as well as in a few blood and swab samples. Moreover, all challenged birds produced TBEV antibodies and showed a mild to severe acute to subacute necrotizing encephalitis. TBEV-specific antigen was detected in the brain of 14 ducks by immunohistochemistry. The short and low viremic phases, as well as the low virus load in tissues, suggest that ducks should not be considered as reservoir hosts. However, due to the high antibody levels, ducks can serve as sentinel species for the detection of natural TBEV foci.

KEYWORDS

arbovirus, ducks, pathogenesis study, tick-borne encephalitis virus

1 | INTRODUCTION

Tick-borne encephalitis virus (TBEV) is a tick-borne *Flavivirus*, which is endemic in many European countries and throughout most of Asia (Mansfield et al., 2009). Originally, three different TBEV subtypes were distinguished: The Western European, the Siberian and the Far Eastern subtype (Valarcher et al., 2015). Just recently, two additional subtypes have been proposed (Dai, Shang, Lu, Yang, & Xu, 2018; Kovalev & Mukhacheva, 2017). TBEV is circulating in small, geographically defined natural foci between the tick, as vector and small mammals such as rodents, as hosts and reservoirs (Dobler, Gniel, Petermann, & Pfeffer, 2012; Dobler, Hufert, Pfeffer, & Essbauer, 2011). In

Europe, TBEV is in most cases transmitted by *Ixodes ricinus*, whereas in Russia and Asia, the most important tick vector is *Ixodes persulcatus* (Ruzek et al., 2019). Non-viremic transmission by co-feeding of infected and non-infected ticks in close proximity is also considered to play a substantial role in maintaining TBEV circulation (Labuda & Randolph, 1999). Moreover suitable environmental, socio-economic and climatic conditions are essential for the establishment of natural foci (Estrada-Peña & de la Fuente, 2014; Korenberg, 2009).

More than 10,000 human cases are reported in Europe and Asia every year (Bogovic & Strle, 2015). Even more, the true number of cases is most probably much higher, as mild cases often remain undiagnosed and since TBE is not in all European countries a notifiable disease (Donoso Mantke, Escadafal, Niedrig, Pfeffer, & Working Group For Tick-Borne Encephalitis Virus, 2011).

For Germany, the number of clinical infections fluctuates around a median of 283 human cases reported every year (Robert-Koch-Institute, 2019). High prevalence areas are Baden-Württemberg and Bavaria with most of the cases (89% from 2001 till 2018) (Hellenbrand et al., 2019). Moreover, several regions in Hesse and Thuringia and isolated districts in Saarland, Rhineland Palatine and Saxony are also TBEV risk areas. In addition, single autochthonous human TBE cases are reported from other federal states caused by the patchy pattern of TBEV natural foci that can be fairly small (Kupča et al., 2010; Robert-Koch-Institute, 2019).

In most cases, TBEV is transmitted by tick bites. However, alimentary infections by the consumption of unpasteurized dairy food products are also possible (Brockmann et al., 2018; Offerdahl, Clancy, & Bloom, 2016). Approximately one third of the infected humans suffer from neurological symptoms, while the majority of infections manifests only in mild non-specific symptoms or is inapparent (Kaiser, 2012). Humans as well as various animal species like dogs, horses, monkeys and ruminants are accidental hosts (Klaus, Hoffmann, Hoffmann, & Beer, 2016). Infections are possible in these species; however, they do probably play only a minor role in the virus transmission cycle.

During the last decades, a geographic expansion, characterized by an increase in the number of high risk areas and the emergence of new natural TBEV foci, was observed in Germany and in various other European countries (Riccardi et al., 2019; Robert-Koch-Institute, 2019). The way of dispersal of TBEV to previously unaffected regions and the establishment of new natural foci is not quite clear. The distribution of infected ticks by wild animals, such as rodents or deer, or even by humans transporting infested animals is a possible option (Boelke et al., 2019). Another possibility is the transport of TBEV infected ticks by birds, which can easily cross rough terrain (Hasle, 2013). Birds are playing an important role in the spread of various arboviruses or their respective vectors, along their major flying routes (Hubálek, 2004; Jourdain, Gauthier-Clerc, Bicout, & Sabatier, 2007). In several European countries, migratory and resident wild birds were screened for TBEV infected ticks; however, the virus prevalence of infested ticks attached on birds seems to vary substantially. For instance, in Latvia 14% (Kazarina et al., 2015) of the ticks were TBEV positive, whereas the prevalence in Sweden (0.53%) (Waldenström et al., 2007) and Germany (0%) (Klaus, Gethmann, et al., 2016) was quite low or non-detectable. Nonetheless, considering the annual migration of billions of birds, infected ticks may still be carried by birds, even if the TBEV prevalence in them is rather low. Mainly nymphs and larvae of *Ixodid* ticks are feeding on birds and need only a short period (2–7 days) for their blood meal (Balashov, 1972). During this short time, birds are flying only short distances, which speak against the long-distance transport of infected ticks (Klaus, Gethmann, et al., 2016). Nevertheless, a spread over shorter distances, for instance from one stopover site to another, is already a progression.

Whether birds are also playing an active role in the transmission cycle of TBEV, apart from their function as mechanical vector, is not fully understood. The available literature regarding animal experiments dates back to the 1960s. A variety of wild and domestic bird species were tested, but most of them did not exhibit a viraemia or showed a seroconversion (Ernek, 1962; Ernek, Kožuch, & Nosek, 1969; Ernek, Kožuch, Nosek, & Hudec, 1969; Ernek & Lichard, 1964; Grešíková & Ernek, 1965; Grešíková, Nosek, Řeháček, & Albrecht, 1962; Nosek, Grešíková, Řeháček, Kožuch, & Albrecht, 1962; Řeháček, Grešíková, Nosek, & Albrecht, 1963; Streissle, 1958; van Tongeren, 1983). Ducks, however, developed a viraemia and seemed to be susceptible for the investigated TBEV strains. Furthermore, the virus was isolated several times from wild ducks and neutralizing antibodies were detected, indicating the possibility of a natural infection (Ernek, 1967, 1975; Ernek, Kožuch, Nosek, Hudec, & Folk, 1975). Mallards (*Anas platyrhynchos*) are usually partial migratory birds, meaning parts of the population stay all year in their habitat and parts migrate, depending on their geographic origin. Ducks from Northern Europe are usually migratory birds, whereas ducks from Central Europe travel over shorter distances or do not migrate (van Toor et al., 2013). A spread of infectious diseases by ducks therefore seems possible. In this study, we challenged domestic ducks with the TBE strain Neudoerfl. The aim was to reveal whether ducks are susceptible for TBEV Neudoerfl and may even function as silent virus carrier.

2 | MATERIALS AND METHODS

2.1 | Animals

Twenty Peking ducks were purchased at the age of 4 weeks and monitored daily for their physical health. Pre-challenge samples were taken and examined by quantitative real-time RT-PCR (qRT-PCR) and virus neutralization test (VNT) to exclude previous infections with TBEV. To clear a *Salmonella* spp. infection, the ducks were treated with Enrofloxacin. The animals were challenged eventually at the age of 6 weeks.

2.2 | Virus challenge

The TBEV animal experiment was carried out under biosafety level three conditions. Nineteen ducks were infected subcutaneously (s.c.) with 10^5 $TCID_{50}$/ml virus dilution of TBEV strain Neudoerfl (GenBank accession no. U27495), and one duck was kept as a negative control. Virus diluted in minimal essential medium (MEM) was injected

subcutaneously in the knee folds of each animal (0.5 ml per side). For control purpose, residual virus solution was back titrated to verify that there was no virus infectivity loss during the challenge procedure.

During the infection experiments, the ducks were examined daily for clinical symptoms following a defined score sheet (scores 0 [no clinical changes] up to 3 [severe disease]). Changes in behaviour, body posture, respiratory symptoms, plumage, feed intake, defecation and the nutritional condition in general were documented daily. Blood samples, oropharyngeal and cloacal swabs were taken on days 0, 2, 4, 6, (8 and 12 just swabs), 10, 14 and 21 post-infection (dpi), and the body weight was measured (additional time points 17 and 19 dpi). Blood samples were centrifuged and the blood cruor and serum aliquoted and stored at −70°C. Swab samples were placed in 2 ml MEM containing antimicrobials (Gentamicin, Amphotericin, Lincomycin, Enrofloxacin) and shaked for 30 min. The supernatant was decanted and stored at −70°C for further examination. After 21 days, the ducks were euthanized and tissue samples (e.g., brain, liver, spleen, heart, *bursa cloacalis*) were taken for virological investigations and for histopathology.

2.3 | Virological analysis

Tissue samples were homogenized in 500 µl MEM with antibiotics (Penicillin/Streptomycin). Supernatants were collected after centrifugation and tested by qRT-PCR. Samples with C_t values below 30 were titrated on PK 15 cells. After 7 days, cell monolayers were formalin-fixed and stained with 1% crystal violet solution to reveal cythopathic effects.

2.4 | Quantitative real-time RT-PCR

RNA of the avian blood samples was isolated from the blood cruor using the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. The RNA of the swab and tissue sample supernatants was extracted using a Biosprint 96 (Qiagen) and the NucleoMag VET Kit® (Macherey-Nagel). An internal control RNA (IC2 RNA) was extracted together with all samples (Hoffmann, Depner, Schirrmeier, & Beer, 2006). Extracted RNA was eventually analysed by a TBEV-specific qRT-PCR using the protocol described by Schwaiger and Cassinotti (2003). A synthetic RNA was used for the quantification of the exact number of genome copies in the swab, blood and organ samples. Copy numbers above three copies/µl RNA were regarded as positive, one to three copies/µl were inconclusive, and less than one copy/µl RNA were negative.

2.5 | Serology

Neutralizing antibody titres against TBEV strain 'Neudoerfl' were determined by a virus neutralization test (VNT) as described before (Ziegler, Angenvoort, Klaus et al., 2013). All samples were run in duplicate, and cytopathic effects were read after 7 days. The neutralizing antibody titre (neutralization dose 50% [ND_{50}]) of a serum sample was defined as the maximum dilution which inhibited cytopathic effects in fifty per cent of the wells and was calculated according to the Behrens-Kaerber method.

Serum antibody levels from 0, 6, 14 and 21 dpi were additionally investigated using a commercially available competition ELISA (Immunozym FSME IgM Kit; Progen GmbH). The samples were analysed by the modified version published by Klaus et al. (2011) to determine total immunoglobulin. The VNT and ELISA results were compared to estimate cut-off values to distinguish positive, inconclusive and negative ELISA results. The upper cut-off value was calculated using a receiver operating characteristic analysis (ROC analysis) with regard to the criterion 'minimum ROC distance' using the software R version 3.6.0—'Planting of a Tree' with the package OptimalCutpoints (López-Ratón, Cadarso-Suárez, Rodríguez-Álvarez, & Gude-Sampedro, 2014; Metz, 1978; R Core Team, 2019). Additionally, a lower cut-off was determined by the mean value plus three standard deviations of the negative controls (Lardeux, Torrico, & Aliaga, 2016).

2.6 | Histopathology and immunohistochemistry

Tissue samples were fixed in neutral buffered formalin (4%), embedded in paraffin and stained with haematoxylin/eosin (H&E). Brain and spleen samples as well as tissues showing histopathological alterations which might have been associated to a TBEV infection were examined by IHC. For this purpose, 3 µm sections were cut, deparaffinized and rehydrated. Endogenous peroxidase was blocked using 3% hydrogen peroxide/methanol followed by a proteinase K digestion step (10 mg/ml) for 15 min at 37°C to retrieve the virus antigen. The primary antibody, a polyclonal rabbit antibody against TBEV, (Center for Virology, Medical University of Vienna, Austria) was used at a dilution of 1:2,000. Negative control sections were incubated only with goat serum. The slides were developed by using Rabbit Envision HRP and Diaminobenzidine (DAB) as substrate. Tissue samples were rated depending on the percentage of positive cells with scores from 0 to 3 (0 = no positive cells/negative; 1 = <1% positive cells = mildly affected; 2 = ≥1% and <5% positive cells = moderately affected; 3 = ≥5% positive cells = severely affected). The IHC slides were looked through from one end to the other end and stained cells were counted.

3 | RESULTS

None of the infected ducks showed TBEV related clinical symptoms and the animals steadily gained weight (data not shown). The clinical score was '0' in all categories during the whole experiment. However, one duck (D 16) had to be euthanized on 8 dpi due to an acute arthritis and erosive pododermatitis, which was not associated with the TBEV infection.

3.1 | TBEV detection by qRT-PCR

TBEV-specific RNA was detected in few blood samples, oropharyngeal and cloacal swab samples with high C_t values, whereas the samples of the remaining infected ducks were negative (Figure 1). TBEV-RNA was detected in the swab samples on day 2 (5/19), 6 (1/19) and 8 (1/19) post-infection (pi). In few blood samples, TBEV-RNA was found on day 4 (2/19) and 6 (3/19) pi. Duck 10 and Duck 19 were virus positive in the blood samples on two following sampling days.

The brain samples of all ducks (19/19) were tested positive for TBEV-RNA, including the brain sample of Duck 16, which was already euthanized after 8 dpi. Additionally, TBEV-RNA was detected in the spleens of few birds (4/19) (Table 1). Brain samples with C_t values below 30 were titrated on cells, but a virus cultivation from the tissue samples on cell culture was not successful.

3.2 | Serological results

All infected ducks seroconverted with high virus neutralizing antibody titres. Neutralizing antibodies were first detected on 6 dpi (17/19). The titres did reach their maximum on day 10 and 14 post-infection and ranged then from 1:960 to 1:20,480 ND_{50} (Figure 2a).

The ROC analysis for the criterion 'minimum ROC distance' estimated a cut-off value of 15.2079 U/L and defined the upper cut-off to 16 U/L. All values above this cut-off were regarded TBEV positive. Additionally, a lower cut-off was calculated to define a range of inconclusive test results between the lower and the upper cut-offs. Based on the median and three deviations of the ELISA results of the negative ducks, investigated before the infection, the lower cut-off was estimated to 0.72262 U/L. Thus, ELISA results ranging between 0.72262 and 16 U/L were regarded as inconclusive. On day 6 post-infection, two VNT negative ducks were inconclusive in the ELISA, while one VNT positive was inconclusive in ELISA. On day 14 and 21 post-infection, the ELISA results are in accordance with the VNT results: All ducks were clearly positive on these days (Figure 2b).

3.3 | Gross lesions, histopathology and immunohistochemistry

The gross examination of the animals revealed no specific lesions indicating a viral disease. In the histopathology, all ducks showed an acute lymphohistiocytic (6/19) or subacute lymphoplasmacellular (13/19) non-suppurative necrotizing encephalitis, and one animal also a meningitis. The alterations were mild (5/19) up to moderate (13/19) or severe (1/19). Cerebrum was involved in all cases, mesencephalon, which was not available in all ducks, was involved to a lesser degree (12/15), so was brain stem (12/19) and cerebellum (11/19). Alterations were seen in white and grey matter and consisted of perivascular lymphohistiocytic or lymphoplasmacellular cuffing (Figure 3a,b), as well as multifocal neuronal and glial single cell necrosis, not in all cases associated with a mild glial and/or inflammatory cell reaction (Figure 3c). These necrotic foci were mainly seen in cerebrum (18/19), less frequent in mesencephalon (3/15), brain stem (3/19) and cerebellum (2/19). Clear signs of degeneration (karyorrhexis) were also seen in perivascular and infiltrated inflammatory cells. Only few glia nodules were seen, mainly in cerebrum (17/19), but also in mesencephalon (7/17), cerebellum (6/17) and brain stem (4/17). Additionally, some animals revealed a mild acute

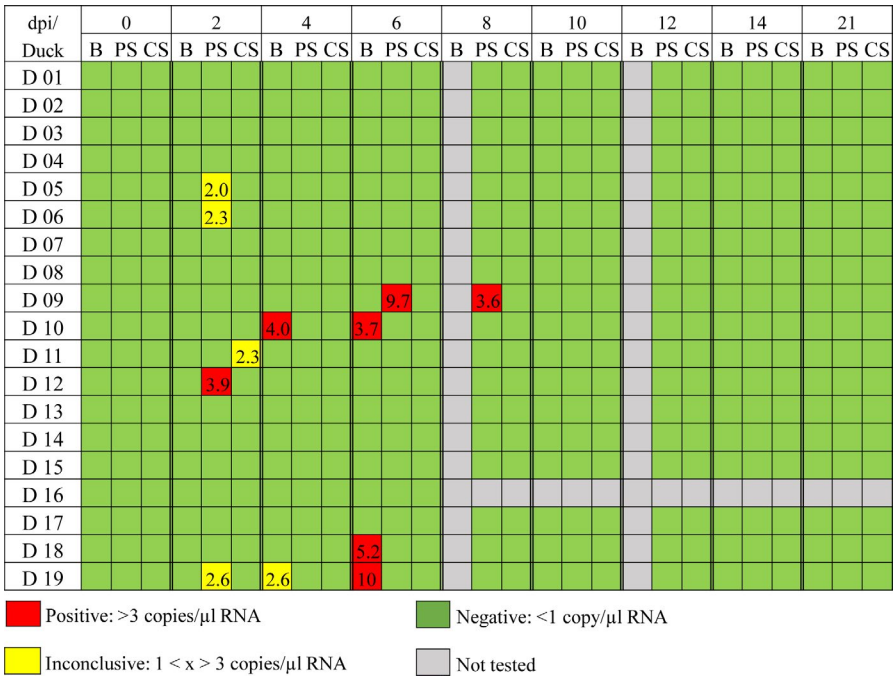

FIGURE 1 Quantitative real-time RT-PCR (qRT-PCR) results of the blood and swab samples of the infected ducks (D 01-D 19) in copies/µl. B, Blood samples; CS, Cloacal Swab; PS, Pharyngeal Swab

TABLE 1 Results of the tissue samples by quantitative real-time RT-PCR (qRT-PCR), titration on PK15 cells and immunohistochemistry (IHC)

Duck	Tissue sample	C_t	cop/µl RNA	log $TCID_{50}$/ml	IHC
D 01	Brain	27.83	551.03	n.v.d.	+
D 01	Spleen	N/A	/	*n.d.*	–
D 02	Brain	28.75	287.90	n.v.d.	+
D 02	Spleen	N/A	/	*n.d.*	–
D 03	Brain	29.16	215.91	n.v.d.	+
D 03	Spleen	35.66	2.27	*n.d.*	–
D 04	Brain	29.46	174.92	n.v.d.	–
D 04	Spleen	N/A	/	*n.d.*	–
D 05	Brain	31.30	48.08	*n.d.*	–
D 05	Spleen	N/A	/	*n.d.*	–
D 06	Brain	29.46	175.22	n.v.d.	+
D 06	Spleen	35.38	2.75	*n.d.*	–
D 07	Brain	31.63	38.34	*n.d.*	+
D 07	Spleen	N/A	/	*n.d.*	–
D 08	Brain	30.60	79.00	*n.d.*	+
D 08	Spleen	N/A	/	*n.d.*	–
D 09	Brain	27.65	626.33	n.v.d.	+
D 09	Spleen	N/A	/	*n.d.*	–
D 10	Brain	31.16	53.33	*n.d.*	+
D 10	Spleen	N/A	/	*n.d.*	–
D 11	Brain	33.49	10.36	*n.d.*	–
D 11	Spleen	N/A	/	*n.d.*	–
D 12	Brain	30.97	60.72	*n.d.*	–
D 12	Spleen	N/A	/	*n.d.*	–
D 13	Brain	32.90	15.67	*n.d.*	–
D 13	Spleen	N/A	/	*n.d.*	–
D 14	Brain	31.23	50.59	*n.d.*	+
D 14	Spleen	N/A	/	*n.d.*	–
D 15	Brain	32.17	26.20	*n.d.*	+
D 15	Spleen	N/A	/	*n.d.*	–
D 16	Brain	29.13	221.28	n.v.d.	+
D 16	Spleen	31.11	55.04	*n.d.*	–
D 17	Brain	31.78	34.51	*n.d.*	+
D 17	Spleen	N/A	/	*n.d.*	–
D 18	Brain	30.70	73.52	n.v.d.	+
D 18	Spleen	32.99	14.71	*n.d.*	–
D 19	Brain	30.87	65.37	n.v.d.	+
D 19	Spleen	N/A	/	*n.d.*	–

Abbreviations: IHC (Immunohistochemistry): +, positive cells; –, negative; N/A, no C_t; *n.d.*, not done; n.v.d., no virus detected.

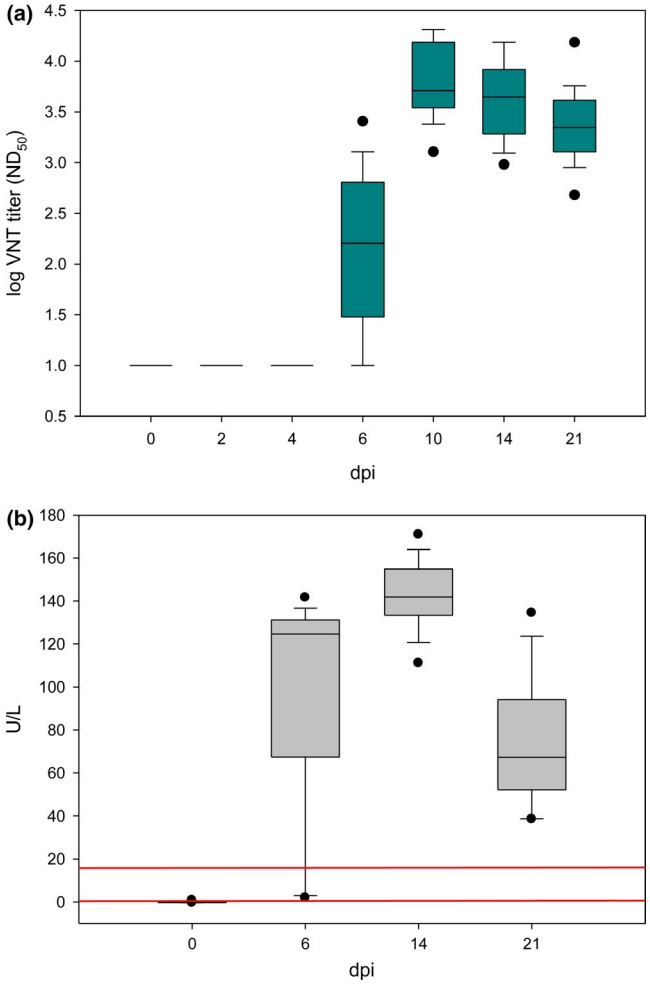

FIGURE 2 Antibody response of the infected ducks against TBEV, by virus neutralization test and ELISA. (a) Antibody response against TBEV, by virus neutralization test (depicted in log titres). Data of the neutralizing antibody response for all ducks are presented in a box plot. Minimum and maximum values are represented by the respective end of the whiskers and outliers as dots. The box includes 50% of the values of all investigated animals per day, and the median is depicted as a line. (b) Total immunoglobulin detected against TBEV by ELISA in units per litre (U/L) on day 6, 14 and 21 post-infection. The cut-off values are depicted as red lines: Samples with <0.72262 U/L were regarded as negative, ≥0.72262 and ≤16 U/L as inconclusive, and >16 U/L as positive. Data of the antibody response for all ducks are presented in a box plot. Minimum and maximum values are represented by the respective end of the whiskers and outliers as dots. The box includes 50% of the values of all investigated animals per day, and the median is depicted as a line

non-suppurative vasculitis (7/19) and nine ducks showed a reactive astrogliosis of varying degree (Figure 3d).

By immunohistochemistry, TBEV viral antigen was detected in the brain of 14 out of 19 infected ducks. All animals showed only a mild accumulation, which was confined to neurons and neuronal processes of the cerebrum (Figure 3e,f). Only one animal revealed a staining reaction in the mesencephalon, too. The infected cells were in parts associated to histopathological alterations of the brain (i.e., perivascular cuffing, foci of necrosis), but randomly distributed positive cells were also seen.

Further alterations of unknown origin were also seen in some ducks. This includes a focal acute non-suppurative vasculitis in the

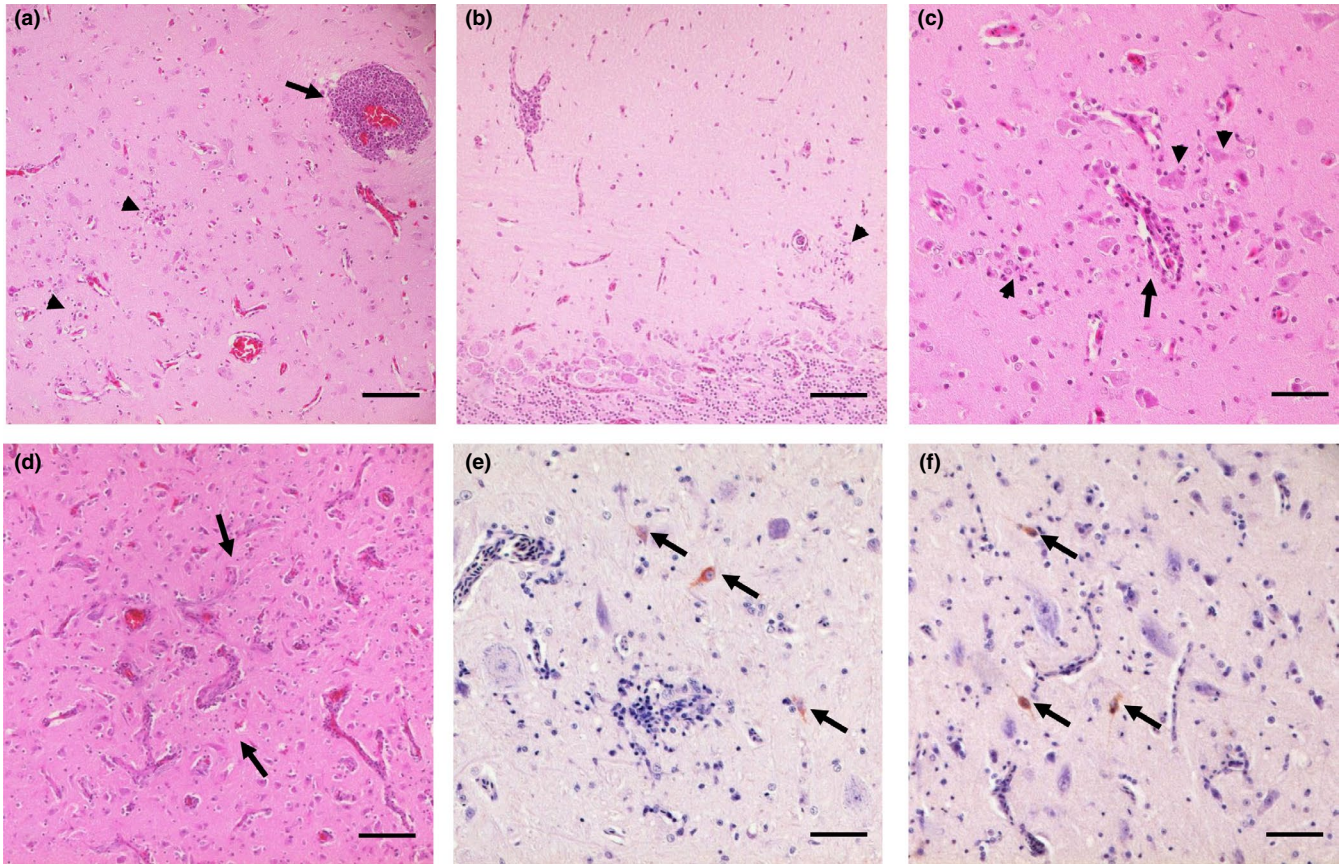

FIGURE 3 Histopathology and immunohistochemistry of TBEV infected ducks. (a) H&E, Duck E04, cerebrum, severe lymphohistiocytic perivascular cuffing (black arrow), gliosis and glial/neuronal single cell necrosis (arrowhead) in adjacent neuropil; (b) H&E, Duck E06, cerebellum, mild perivascular cuffing and glia nodule (arrowhead); (c) H&E, Duck E04, cerebrum, mild lymphohistiocytic perivascular cuffing with signs of degeneration (black arrow) as well as glial/neuronal necrosis (arrowhead) in adjacent neuropil; (d) H&E, Duck E13, cerebrum, focal reactive astrogliosis (black arrow); (e, f) Immunohistochemistry (polyclonal antibody anti TBEV), Duck E06 and E14, cerebrum, lesion associated neuronal detection of TBEV antigen (black arrow); (a–c): bar 50 µm, (d–f): bar 20 µm

gut wall of one animal as well as a follicular hyperplasia of the spleen in six ducks. Viral antigen was not detectable. Additionally, in several animals, lesions were seen, which were most probably due to an unrelated bacterial or parasitic aetiology.

No comparable alterations were seen with the negative control animal.

Table S1 summarizes these additional diagnoses.

4 | DISCUSSION

Tick-borne encephalitis has become a growing health problem in endemic European and Asian countries with a global increase in human cases (Beauté, Spiteri, Warns-Petit, & Zeller, 2018; Lundkvist, Wallensten, Vene, & Hjertqvist, 2011; Velay et al., 2018). Multiple different factors like the weather, environmental changes and host abundance, but also the growing awareness of the health authorities and improved diagnostics, are playing a role in the increased incidence of TBEV (and its tick vector) during the last decades (Petri, Gniel, & Zent, 2010; Randolph, 2010). Furthermore, an expansion of the risk areas has been observed and new natural foci/ endemic areas have emerged (Beauté et al., 2018; de Graaf et al., 2016). The background for the new appearance of natural foci is currently under extensive discussion. The possible role of birds in the spread of ticks and tick-borne pathogens, like TBEV, is not yet fully elucidated.

Apart from being a possible mechanical vector for infected ticks, birds may represent a potential virus reservoir for TBEV. Infection experiments with various bird species, different TBEV strains and inoculation methods were already conducted about sixty years ago—with all limitations of then available diagnostic technologies: Infected Great tits (*Parus major*), House sparrows (*Passer domesticus*), Pheasants (*Phasanius colchicus*), Common buzzards (*Buteo buteo*) and European kestrels (*Falco tinnunculus*) did not develop clinical symptoms or a viraemia, and only occasionally neutralizing antibodies were detected (Ernek & Lichard, 1964; Grešíková et al., 1962; Nosek et al., 1962; Řeháček et al., 1963). Common coots (*Fulica atra*) and chickens (*Gallus gallus domesticus*), however, showed a viraemia but no clinical symptoms (Streissle, 1958; van Tongeren & Timmers, 1961). Animal experiments with different TBEV strains were conducted on wild and domestic ducks (*Anas platyrhynchos/ Anas platyrhynchos domesticus*) between the 1960s and 1980s (Ernek, 1962; Ernek, Kožuch, & Nosek, 1969; Ernek, Kožuch, Nosek, et al., 1969; van Tongeren, 1983).

In these experiments, ducks were infected with TBEV homogenized mouse brain tissue of strain Hypr or strain Graz I. In all experiments, a viraemia lasting over several days and seroconversion was seen.

As experimentally infected ducks seemed to be susceptible to the TBEV strains Hypr and Graz I in principle, we decided to investigate their susceptibility to the TBEV strain Neudoerfl, representing the prototype of the European subtype, which was not tested before. Another reason is that mallards (*Anas platyrhynchos*) are partial migratory birds, enabling the transport of infected ticks. The aim of this study was to find out whether ducks can be productively infected with TBEV strain Neudoerfl.

In the here presented animal trial, no clinical symptoms were observed among the infected ducks, which is in accordance with the duck experiments in the past. In comparison to the challenge study with TBEV strain Hypr and Graz I conducted by Ernek et al. and van Tongeren, where the majority of the ducks developed a viraemia which lasted four to five days, TBEV-RNA was detected only sporadically in the blood of a few ducks in the present study. Differences in the neuropathogenicity of different strains within the European subtype are known: TBEV Neudoerfl has a low neuropathogenicity, whereas the neuropathogenicity, after an infection with TBEV Hypr, is higher (Dobler, Bestehorn, Antwerpen, & Overby-Wernstedt, 2016).

Although TBEV-RNA was detected only in a few ducks in the blood, all infected ducks of the here described animal experiment developed neutralizing antibodies. A seroconversion was detected early (on 6 dpi) in some ducks; thus, it may not be possible to form a prolonged viremia, as the virus was removed too rapidly from the bloodstream. Remarkably, neutralizing antibody levels reached very high titres with up to 20,480 ND_{50}, indicating a strong stimulation of the immune system. It is not possible to compare the antibody titres with these of the animal experiments conducted on ducks in the past, as methodological details used were different or unknown.

Interestingly, TBEV-specific RNA was detected in the brain samples of all infected ducks, albeit it was impossible at any time point to re-isolate virus. It is possible that viral loads in the organ samples were too low or the detected virus was not viable.

The histopathological observation of a non-suppurative encephalitis with distinct neuronal necrosis, foci of neuronophagia, gliosis and perivascular lymphohistiocytic or lymphoplasmacellular cuffing is largely in accordance with the neuropathology described for mammals (Bagó, Bauder, Kolodziejek, Nowotny, & Weissenböck, 2002; Böhm et al., 2017; Süss et al., 2007; Völker, Hoffmann, Nessler, Baumgärtner, & Wohlsein, 2017; Weissenböck, Suchy, & Holzmann, 1998). However, in birds, the involvement of the meninges was rare. Additionally, the main target region in birds seemed to be the cerebrum, followed by mesencephalon, cerebellum and brain stem. This distribution of lesions shows some resemblance to dogs (Völker et al., 2017), with a decreasing intensity from cranial to caudal. Interestingly, several birds displayed signs of a mild vasculitis, which is frequently observed in birds infected with West Nile virus, a closely related Flavivirus (Ziegler, Angenvoort, Fischer et al., 2013). Furthermore, a reactive astrogliosis seen in several animals indicated the beginning glial scar formation due to a previous severe tissue damage. Even more interestingly were the distinct signs of the degeneration in glial and mononuclear inflammatory cells within the perivascular cuffs and throughout the neuropil. These lesions are described by others (Böhm et al., 2017; Weissenböck et al., 1998). Additionally, a distinct number of granzyme B releasing cells were detectable in TBEV infections in monkeys (Süss et al., 2008) and humans (Gelpi et al., 2005, 2006), indicating an, at least partial, involvement of immunopathological processes for some of the tissue damage. Therefore, future studies are needed to further investigate whether these signs of karyorrhexis are due to viral or cytokine induced apoptosis or necrosis. However, viral antigen was not detected in glial cells, glial nodules or in foci of acute neuronophagia. A distinct staining reaction was only seen in neurons and neuronal processes, which were often closely associated to inflammatory processes, but never in the centre of it. As described before, only a small amount of viral antigen was found, if at all (Böhm et al., 2017; Völker et al., 2017; Weissenböck et al., 1998). This is not a surprise as all birds were killed at 20 or 21 dpi. Flavivirus infections such as TBEV in humans and dogs and WNV in birds are rapidly cleared; hence, there is only a small window for an antigen detection (Angenvoort et al., 2014; Weissenböck et al., 1998). There is a divergence between the widespread histopathological lesions throughout all regions of the brain and the locally restricted viral antigen detection only in the cerebrum (except for one detection in the mesencephalon). Such a pattern was also described in a monkey before (Süss et al., 2008).

Results regarding histopathology and seroconversion were similar to historical reports (Ernek, 1962; Ernek et al., 1969; Ernek et al., 1969; van Tongeren, 1983). However, a high and prolonged viraemia like in the previous infection studies with ducks has not been observed, which may be due to the lower virulence of the TBEV strain Neudoerfl. Therefore, ducks do not play a role as an undetected virus reservoir in the ongoing TBEV endemic.

Natural foci for TBEV are often found by tick flagging followed by molecular testing. Serosurveillance of sentinel animals (e.g., sheep and goats) is an alternative approach to identify TBEV foci (Klaus et al., 2012). According to Komar (2001), the perfect sentinel species is susceptible to the infection, with rapid seroconversion, yet not developing a clinical disease. Furthermore, the sentinel should not contribute to the local pathogen transmission. Our animal experiments show that ducks fulfil these criteria. Ducks are often kept in free-range husbandry thereby coming in contact with ticks. Monitoring the presence of neutralizing antibodies at the time of slaughter is feasible. The investigation of these ducks in addition to the monitoring of ticks could help to define the distribution/occurrence of TBEV in affected areas or (even help) to detect new natural foci.

5 | CONCLUSION

The duck challenge experiments show their susceptibility to TBEV strain Neudoerfl. However, as ducks did not develop an extended

viremia, they are neither a reservoir nor amplification host, hence do not play a role in the transmission cycle of this virus. However, ducks develop high antibody levels after an infection with TBEV and may therefore be used as sentinels to detect new natural foci.

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ETHICAL APPROVAL

The duck infection experiments described in this publication were approved by the State Office of Agriculture, Food safety, and Fishery in Mecklenburg-Western Pomerania, Germany on the basis of national and European legislation in particular directive 2010/63/EU (Reference number 7221.3-1-075/16).

CONFLICT OF INTEREST

All authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the main manuscript and in the supplementary material of this article.

ORCID

Ute Ziegler https://orcid.org/0000-0001-5295-7339
Martin Eiden https://orcid.org/0000-0002-1197-8288
Martin H. Groschup https://orcid.org/0000-0003-0215-185X

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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