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# Survey on viral pathogens in wild red foxes (*Vulpes vulpes*) in Germany with emphasis on parvoviruses and analysis of a DNA sequence from a red fox parvovirus

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## SUMMARY

The seroprevalence of canine parvovirus (CPV), canine distemper virus (CDV), canine adenovirus (CAV) and canine herpesvirus (CHV) infections in red foxes (*Vulpes vulpes*) was determined in fox sera collected between 1991 and 1995. A total of 500 sera were selected and the seroprevalences were estimated to be 13% (65 of 500 sera) for CPV, 4·4% (17 of 383 sera) for CDV, 3·5% (17 of 485 sera) for CAV, and 0·4% (2 of 485 sera) for CHV, respectively. No statistically significant differences were observed between the two (rural and suburban) areas under study.

Parvovirus DNA sequences were amplified from tissues of free-ranging foxes and compared to those of prototype viruses from dogs and cats. We report here a parvovirus sequence indicative of a true intermediate between the feline panleukopenia virus-like viruses and the canine parvovirus-like viruses. The red fox parvoviral sequence, therefore, appears to represent a link between those viral groups. The DNA sequence together with a significant seroprevalence of parvovirus infections in foxes supports the hypothesis that the sudden emergence of canine parvovirus in the domestic dog population may have involved the interspecies transmission between wild and domestic carnivores.

## INTRODUCTION

The feline parvovirus subgroup comprises viruses that are important pathogens for their carnivore hosts. Viruses comprising this group have been isolated from a variety of carnivore species and named accordingly, hence the names mink enteritis virus (MEV), feline panleukopenia virus (FPV), canine parvovirus (CPV), raccoon parvovirus (RPV), raccoon dog parvovirus (RD), and blue fox parvovirus (BFPV); for review see [1]. All members of the group are very closely related, with $> 98\%$ genetic homology, but they differ in important biological properties such as natural host range or tissue tropism of replication in disparate hosts. The true relationships have not been determined for all members of the group, but it is clear that the canine and raccoon dog parvoviruses are distinct from the viruses of cats, minks and raccoons [2, 3]. These viruses are of particular importance not only as the cause of serious disease, but they also represent an exemplary system to study viral evolution and to examine the role of interspecies transmission in the emergence of new pathogens.

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Canine parvovirus emerged as a new viral pathogen of dogs in the late 1970s, most likely as a variant of the long known FPV or a closely related parvovirus. Extensive genetic analysis of numerous CPV and FPV isolates indicate that the acquisition of only 3 amino acid changes in the capsid protein of this structurally simple virus would have been sufficient to generate a virus with biological properties characteristic of the novel canine virus [2, 4]. Those analyses also revealed that all CPV isolates studied acquired additional changes during the adaptation of the progenitor virus to CPV, that abolished its ability to replicate in cats [5, 6]. The host range of the progenitor virus is not known, but the original CPV virus failed to replicate in cats. All CPV isolates differ consistently from all FPV/MEV isolates studied by 6 amino acid changes, ones that are responsible for principal biological differences, such as host range and antigenicity [2, 7].

Several hypotheses have been proposed to account for the emergence of CPV, including the involvement of modified-live FPV vaccine strains or the gradual accumulation of those 6 amino acid changes. The sudden appearance of CPV several decades after FPV had been described in cats and mink (*Mustela vison*), as well as epidemiological hints, led to the hypothesis that a carnivore other than the cat may have harboured the direct ancestor of CPV-2. The earliest evidence of CPV-2 in the domestic dog population comes from serological studies in Belgium, where specific antibodies were detected in European dogs in 1976 [8]. The DNA sequence of a parvovirus isolated in Finland from a farmed blue fox (*Alopex lagopus*) revealed non-coding differences that indicated a closer relationship to the CPV-like viruses than any other FPV-like virus studied; however, it was clearly a FPV-like virus by amino acid sequence analysis [2].

Based on those studies, a hypothesis was put forward that the red fox may have harboured an ancestor of CPV, and that such a virus was first transmitted to dogs in Europe. To investigate that hypothesis, we determined the seroprevalence of parvovirus infections in free-ranging red foxes (*Vulpes vulpes*) in Germany and sought to isolate parvoviruses from tissues of free-ranging foxes. In addition, we endeavored to amplify parvovirus DNA by polymerase chain reaction (PCR).

Since the fox sera were made available for the parvovirus study, we extended the serological survey to other viruses of dogs, including canine distemper virus (CDV), canine adenoviruses (CAV), and canine herpesvirus (CHV).

## METHODS

### Geographic areas under study

We compared 2 regions with different wild or domestic carnivore population densities in order to assess the possibility of an interspecies transmission between domestic carnivores and red foxes. The desktop mapping software 'RegioGraph, *vs*. 2.0 (Macon Markt und Konzept, Waghaeusel, Germany) was used to determine study areas of nearly equal size.

We selected all counties within a 15 km range around Berlin (5217–5249 N and 1257–1354 E) as a suburban study population. Berlin, including its suburban counties, has a population of about 4 million people and, therefore, is likely to have a high domestic carnivore population. This area was compared to a predominantly rural area, the district Prignitz, situated in the Northwest of the German Federal State of Brandenburg (5252–5322 N and 1132–1217 E) with a low-density human population (103 000 people) and, presumably, a very low domestic carnivore population. The suburban and rural areas under study covered, respectively, 2534·9 and 2122·9 km². The minimal distance between the two study areas was 80 km (Fig. 1).

*Sera* Fox sera which originated from hunting areas in Brandenburg were collected between January 1991 and December 1995 by hunters and local veterinarians during studies on oral rabies vaccination (suburban area), or the prevalence of the fox tapeworm (*Echinococcus multilocularis*) in red foxes (rural area).

It was not possible to determine the precise number of free-ranging red foxes in those areas; therefore, the number of serum samples allowing statistically significant results were calculated for populations with infinite densities [9]. We accepted 1% ($P < 0{\cdot}05$) as the lowest detectable prevalence and the minimum sample size was determined as 250 fox sera for each study. Fox serum samples that had been stored in the wildlife serum bank of the Federal Research Center for Viral Diseases of Animals, Wusterhausen, were randomly selected from the 922 and 1228 fox sera for the suburban and the rural area, respectively.

### Serology

For the detection of parvovirus specific antibodies, a haemagglutination inhibition (HI) test was performed as described [10]. Briefly, sera were inactivated for 30 min at 56 °C, diluted 1:5 in barbital-borate

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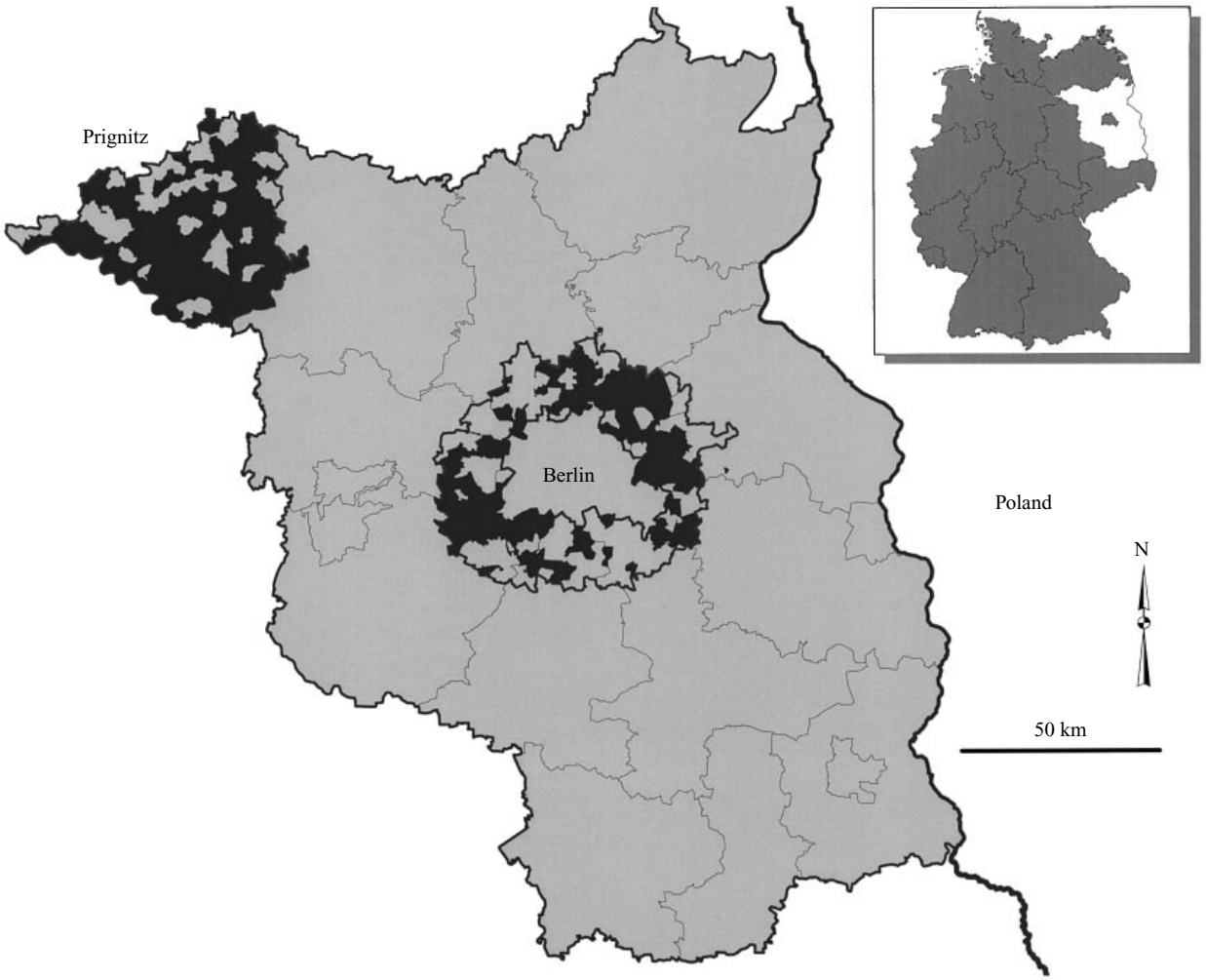


**Fig. 1.** Geographic map of the federal state 'Brandenburg', Germany, and location of the two areas surveyed. Black colour indicates those municipalities within study areas from which fox sera were chosen by random sampling.

albumin buffer, pH 6·2 (BBS), pre-adsorbed to pig erythrocytes and then titrated in BBS. The sera were incubated with 8 haemagglutinating units of CPV for 1 h at room temperature before adding 0·5% pig erythrocytes, and then incubated at 4 °C for 3 h. HI titres $> 10$ were considered positive.

An indirect immunofluroescence antibody test (IFAT) was used to screen fox sera for antibodies against CAV and CHV [1]. A FITC-labelled goat-anti-dog serum (Serva) was used as the conjugate, and all IFAT-positive fox sera were also tested by serum neutralization tests (SNT) using Madin Darby canine kidney cells (MDCK) and 100 $TCID_{50}$ CAV-2 or CHV, as described [12]. Antibodies against CDV were determined in a SNT on Vero cells and 100 $TCID_{50}$ of the CDV-strain 'Onderstepoort' as described elsewhere [13]. Tests were read after 96 h of incubation. Only 383 sera could be examined in this test as the other sera had cytotoxic activity. Negative and positive control sera originated from experimentally infected and uninfected specific-pathogen free dogs. All sera were inactivated for 30 min at 56 °C and diluted 1:10 in phosphate buffered saline before testing.

## Statistics

Fisher's exact test was applied to evaluate differences in antibody prevalence between the 2 study areas using the software Epi-Info Version 6.03 (January 1996, Center for Disease Control and Prevention, Epidemiology Program Office, Atlanta, Georgia, USA). The 95%-confidence intervals (CI) for the determination of the true seroprevalence within the red fox population (CPV: $n = 500$; CAV: $n = 485$;

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Table 1. Antibodies in red foxes to selected viral pathogens based on different assays in two study areas. The 95%-confidence intervals (CI) of true seroprevalences are shown. HI: hemagglutination inhibition test; IFAT: immunofluorescence test; SNT: serum neutralization test

| | | Canine parvovirus (CPV-2) | Canine adenovirus (CAV-2) | Canine herpes virus (CHV) | Canine distemper virus (CDV) |
|---|---|---|---|---|---|
| Test | | HI | IFAT | IFAT | SNT |
| Rural area | Positive | 27/250 | 8/239 | 0/239 | 13/200 |
| | prevalence | 10·8% | 3·34% | — | 6·5% |
| | 95% CI | 7·3–6·49 | 1·5–6·49 | — | 3·6–10·7 |
| Suburban area | Positive | 38/250 | 9/246 | 2/246 | 4/183 |
| | prevalence | 15·2% | 3·65% | 0·81% | 2·1% |
| | 95% CI | 11·4–19·8 | 1·0–7·06 | 0–2·9 | 0·1–6·41 |
| Total | Positive | 65/500 | 17/485 | 2/485 | 17/383 |
| | prevalence | 13·0% | 3·5% | 0·41% | 4·43% |
| | 95% CI | 10·3–15·8 | 2·1–5·2 | 0·1–1·4 | 2·6–7·04 |

CHV: $n = 485$; CDV: $n = 383$) were calculated according to the method of Willer [14]. The significance level was set at $P < 0{\cdot}05$.

### Virus isolation and polymerase chain reaction

Isolation of parvovirus was attempted at the time of necropsy from tissues obtained from red foxes during a study of the prevalence of the fox tapeworm *Echinococcus multilocularis*. Twenty-four sections of the small intestine were collected from foxes with signs of acute gastroenteritis in the areas under study. To increase the probability of parvovirus isolation, 27 tissues from foxes hunted in adjacent counties were included. All tissues ($n = 51$) were triturated, extracted with 10% chloroform, centrifuged at 4% at low speed, and inoculated on Crandell feline kidney cells (CRFK [15]) and canine A72 cells [16]. Cells were cultured for three blind passages, and the supernatants were then tested for parvovirus HA, as described [10].

Tissue samples were examined in parallel for viral DNA by PCR, essentially as described [17]. The primers used were designed to amplify the amino terminal region of the capsid protein gene. The sequence of the primers and their position in the viral genome were:

*Primer M1*: 5′-AGC TGT CGA AAA CGG ATG GGT GGA AAT-3′ (nts 2949–3020).

*Primer 41*: 5′-GCC CTT GTG TAG ACG C-3′ (nts 3825–3840), giving an amplicon of 891 bp.

Amplified DNA was cloned into the vector pCR2.1 (Invitrogen) and sequenced using Taq polymerase on an automated sequencer (Applied Biosystems).

## RESULTS

### Serology

The survey revealed that parvovirus infections are common among red foxes in Germany. Antibodies could be demonstrated in 65 out of 500 sera (13%) by HI tests, with titres ranging from 10 to 640; the majority of sera (80% of total sera) had titres > 40 (Table 1).

Seventeen (3·5%) and 2 (0·4%) of 485 sera were positive in the indirect FAT for CAV or CHV specific antibodies. However, when tested in neutralization tests against CAV-2 only one serum had virus neutralizing activity, with a titre of 80. The sera positive for CHV by the IFAT from the Berlin area were negative when tested for virus neutralizing antibodies. One hundred and seventeen sera could not be used in the SNT against CDV as they were cytotoxic for Vero cells. Seventeen out of 383 sera (4·4%) had neutralizing antibodies against CDV, with titres ranging from 20 to 220 (Table 1). No significant differences in the proportions of seropositive individuals between sampling locations were seen for any of the viruses tested (Table 1).

Multiple infections were rare, for only 2 sera were positive for both CAV and CHV, or CAV and CPV,

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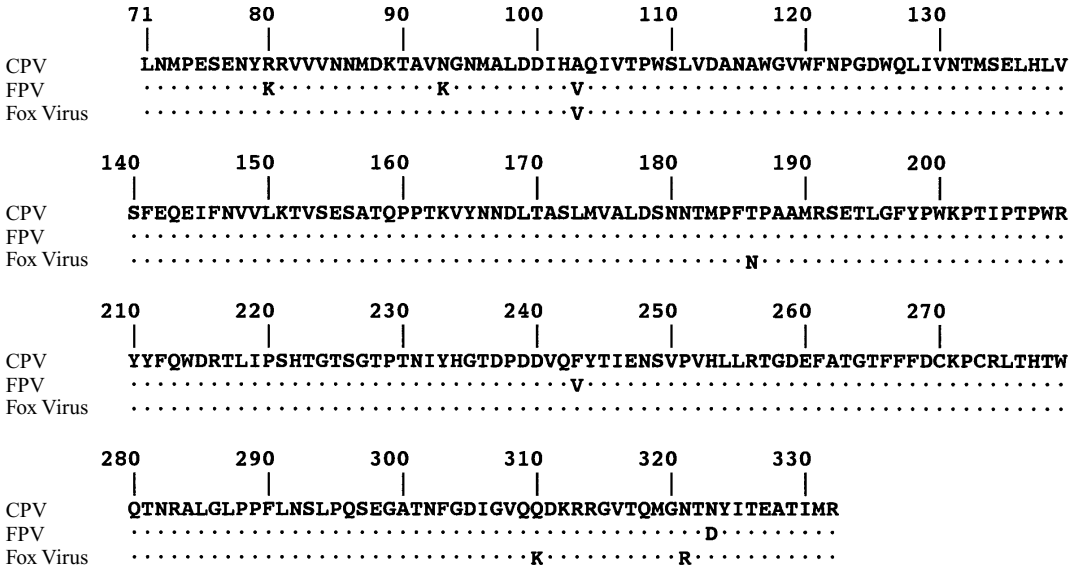


**Fig. 2.** Alignment of amino acids 71–330 of FPV, CPV and the fox virus sequence. The amino acid differences at positions 80, 93, 103 and 323 are conserved among all FPV- and CPV-like viruses. The differences at amino acids 186, 243, 311 and 321 are isolate-specific. (One-letter amino acid code is used.)

or CDV and CPV, respectively. One serum had antibodies against CAV and CPV.

### Virus isolation and polymerase chain reaction

Parvovirus was not isolated in CRFK or A72 cells from any of the 51 faecal samples or gut tissues examined. However, parvoviral DNA sequences were amplified by PCR from two foxes from a geographical region adjacent to the suburban area in the East. One of those amplicons was cloned and sequenced and it revealed a parvovirus sequence that resembled most closely CPV-2, with four nucleotide differences found at nucleotides 3094, 3343, 3714 and 3744. The nucleotide exchanges at position 3094 resulted in a change of amino acid 103 giving the FPV-type sequence at that position, and the changes at nts 3343, 3714 and 3744 gave fox isolate specific changes of amino acids 186, 310 and 321, respectively (Fig. 2).

## DISCUSSION

The main aim of this study was to examine the seroprevalence of parvovirus infections in a European red fox population. This was based on the hypothesis that CPV most likely emerged in Europe and that the red fox may have been the host of an ancestral canine parvovirus. The study was designed in a way that we could compare fox populations in suburban areas with those in more rural areas which should allow us to estimate the possibility of interspecies transmission among domestic and wild carnivores. An average estimate of 13% seropositive foxes in a random population indicates that parvovirus is widespread among red foxes in Germany. This confirms and extends results from Schewers and colleagues [18], who described a 5% seroprevalence rate for fox parvoviruses in France in 1982–4.

Parvoviruses are very stable viruses which are shed in high titres in the faeces during the acute phase of disease. The virus remains infectious for several months in the environment, and contact between foxes and faeces of dogs and cats, and *vice versa*, is likely. Interspecies transmission remains speculative, however, as it is not known whether the fox parvovirus can infect domestic carnivores. If analysed with respect to the geographical origin, the samples from the rural area had an estimated prevalence of 10·8%; however, the samples collected in the suburban area had an estimated prevalence of 15·2% (Table 1). Although the absolute number of positive sera suggests a somewhat higher seroprevalence rate in the suburban area, the difference between the two areas was not statistically significant ($P < 0{\cdot}05$); however, the relatively high seroprevalence and the particular epidemiological features of parvovirus infection, i.e., the copious shedding of a highly stable virus, makes inter-species transmission feasible. HI antibody titres were generally low, with a maximal titre of 640. Post-infection sera in cats and dogs infected with the homologous virus generally have much higher titres –

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usually > 1000, but results vary between laboratories. Reasons for the discrepancies are not known; however, they may be due to poor condition of the sera, or parvovirus infection of red foxes may be different from that of domestic carnivores. Parvovirus disease has not been reported in red foxes, and experimental infections of red foxes with CPV or FPV revealed limited virus replication, although pathological examinations or systematic virus isolation attempts were not performed [19, 20].

Indirect evidence of transmission was obtained by analysis of a parvovirus DNA sequence amplified from small intestinal tissues from a fox. The fox was hunted in the district Prignitz and no clinical record was available. The intestine was examined within the framework of fox tapeworm surveillance and signs of an acute gastroenteritis were found. No histopathological examination was performed. The virus sequence amplified from DNA extracted from those tissues was found to be intermediate between the classical FPV-like viruses and the CPV-like viruses. From the 30% of the capsid protein gene analysed, the virus was most closely related to a CPV type-2 virus, as it has the CPV-specific amino acids 93 and 323. Those amino acids are necessary to give these viruses the CPV-specific antigenic epitope [4]. Three fox virus specific amino acid changes were evident compared to the prototype CPV sequence. Two of these amino acids (aa 310 and 321) are located at the surface of the viral capsid at the threefold spike, a region that represents antigenic epitopes of the virus, including FPV-and CPV-specific epitopes [4]. This implies that the red fox virus may have a different antigenic profile than the canine virus. Unique to the fox virus, however, was the selected exchange of amino acid 103. This amino acid is an important marker for FPV-like viruses, as it is conserved among all the FPV-like viruses examined thus far (Fig. 2). A virus with this intermediate amino acid sequence has not been described, and it appears unlikely that the sequence is due to laboratory contamination or a PCR artifact. The supposition that a fox virus is the direct ancestor of CPV was initially based on the only known fox parvovirus sequence from a Finnish blue fox [2]. That virus had nucleotide sequence similarity to CPV, the amino acid sequences, however, indicated that the virus was a true FPV virus. Our red fox virus sequence, in contrast, represents a true intermediate between the FPV- and CPV-like viruses on the basis of amino acid sequences and supports our hypothesis of a fox parvovirus as a possible ancestor of canine parvovirus. It will be important to examine sequences from additional red fox parvovirus isolates, especially free-ranging foxes.

This study revealed a low estimated seroprevalence (0·4%) of CHV infections in red foxes. The two positive samples may have been due to antibodies which cross-react with CHV since the IFAT was used. Our results conform to studies by others of fox populations in different geographic regions [21].

We also found a low seroprevalence (3·5%) for CAV when tested by the IFAT; however, only one serum (0·25%) had neutralizing activity when tested in the SNT. Our results differed from studies of other fox species and populations; e.g., seroprevalences close to 100% were reported from kit foxes (*Vulpes macrotis mutica*) (16%) [22] or grey foxes in California [23]. The low seroprevalence rates reported here may be important in considering vaccine strategies, since adenoviruses have been employed recently as vectors for genetically engineered oral rabies vaccine for foxes and skunks [24, 25]. Such vaccines would be expected to have different efficacies in different fox populations, depending upon the serostatus of the population.

Based on neutralization assays it appears that CHV and CAV are not significant pathogens of foxes in the areas studied.

CDV infections appear to be widespread among wild carnivores and have been reported in several species, including African wild dogs (*Lycaon pictus*), wolfs (*Canis lupus*), raccoons (*Procyon lotor*), jackals (*Canis mesomelas*), stone martens (*Martes foina*) and, more recently, lions (*Panthera leo*) [26–31]. Although the grey fox is known to be highly susceptible to CDV infection [21, 32], serological evidence for CDV infections have not been reported in grey foxes or island foxes (*Urocyon littoralis*) [21, 23]. However, CDV antibodies have been demonstrated in red fox populations in Spain [32]. Studies on the prevalence of CDV in Germany are limited. Immunohistochemical examination of 90 fox brains did not reveal any positive CDV cases [31]; a serological study with free-ranging foxes from the urban and suburban regions of Berlin, however, revealed a seroprevalence of about 11% [33]. Our results also demonstrated that CDV is present in the German fox populations.

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