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Absence of Antibodies to Foot-and-Mouth Disease Virus in Free-ranging Roe Deer from Selected Areas of Germany (2001–2002)

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ABSTRACT: Blood samples (n = 223) of free-ranging roe deer (*Capreolus capreolus*) were collected from selected hunting grounds in Germany between October 2001 and October 2002. Samples originated from Lower Saxony (n=43) and North-Rhine Westphalia (n=108) within a 20-km area ("cordon") cordoned off along the border of The Netherlands. This is adjacent to the area of a foot-and-mouth disease outbreak that occurred between 21 March and 22 April 2001 in The Netherlands. Negative control samples were taken from northern Germany (Schleswig-Holstein, n=72). Two different enzyme-linked immunosorbent assays (ELISAs) were used for the detection of antibodies against foot-and-mouth disease virus (FMDV) serotype O strain Manisa. To confirm ELISA-positive results, a virus neutralization test was performed. All samples tested negative for antibodies against FMDV. These results suggest that FMDV was not transmitted to free-ranging roe deer living in parts of Germany adjacent to the area affected by the 2001 foot-and-mouth disease outbreak in The Netherlands.

Key words: *Capreolus capreolus*, ELISA, enzyme-linked immunosorbent assay, foot-and-mouth disease virus, Germany, roe deer, serologic survey.

Foot-and-mouth disease virus (FMDV) is classified within the genus *Aphthovirus* in the family *Picornaviridae* and is the etiologic agent of a serious infectious disease that can affect both domestic and free-living Artiodactyla (King et al., 2000; Domingo et al., 2002). Seven serotypes of FMDV with indistinguishable clinical effects occur: O, A, C, South African Territories (SAT) 1, SAT 2, SAT 3, and Asia 1 (Alexandersen et al., 2003). Foot-and-mouth disease (FMD) is a highly contagious acute febrile disease characterized by the formation of vesicles on mucous membranes of the mouth and nose and on hairless skin of the feet. Economically, it is the most important animal disease worldwide (Bachrach, 1968; Domingo et al., 2002). Natural infection has been reported from the families Bovidae, Cervidae, Suidae, Tayasuidae, Camelidae, Giraffidae, Erinaceidae, Muridae, Elephantidae, Tapiridae, and Ursidae (Federer, 1969; Hedger, 1981; Thomson et al., 2001). Morbidity rate can approach 100% (Röhrer and Olechnowitz, 1980). Mortality rates are generally below 5% but can be higher in young animals (Domingo et al., 1990). Foot-and-mouth disease virus can be transmitted directly through close contact with acutely infected animals, by feeding of contaminated animal products, by indirect contact with fomites, and by windborne spread (Alexandersen et al., 2003).

Roe deer (*Capreolus capreolus*) are susceptible to natural and experimental infection with FMDV (Cohrs and Weber-Springe, 1939; Sallinger, 1939; Stroh, 1939; Forman and Gibbs, 1974; Forman et al., 1974), and mutual transmission between roe deer and livestock has been described by Forman et al. (1974) and Gibbs et al. (1975). These experimental studies have included the O and C serotypes. In ruminants, the most common route of infection is via the respiratory tract by inhalation of airborne virus (Korn, 1957). Furthermore, infection via the alimentary tract is possible, although greater doses of virus are required (Sellers, 1971). Roe deer share grazing areas with livestock and

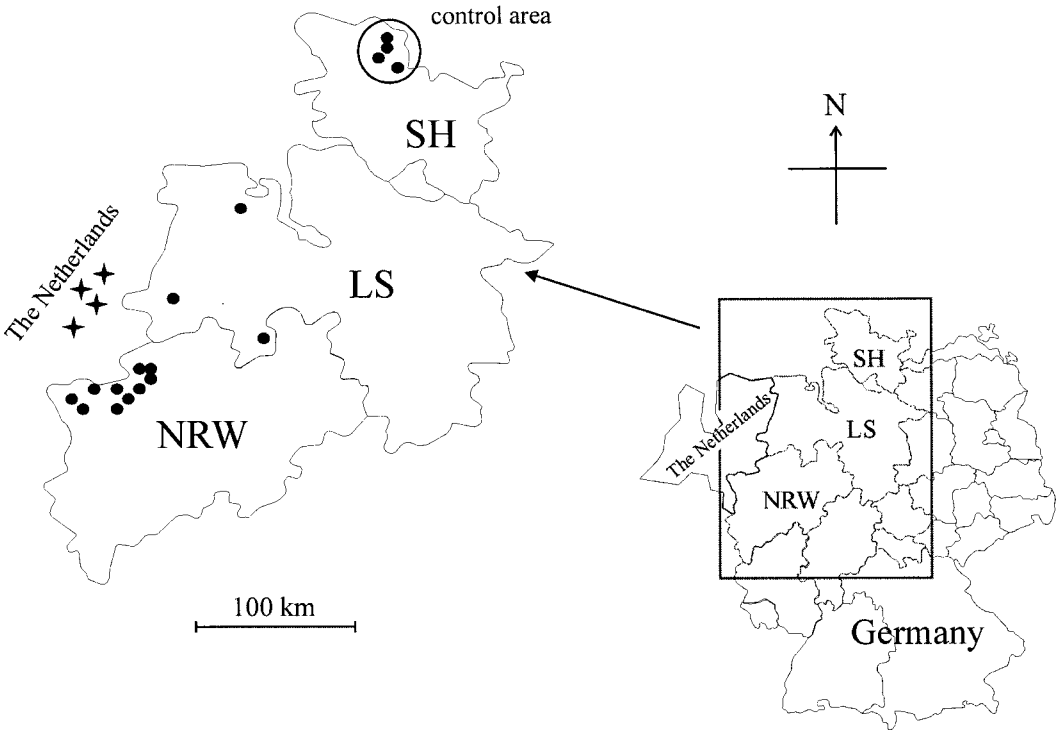

Figure 1. Hunting areas (●) in which blood samples were collected. Samples originated from Lower Saxony (LS) and North-Rhine Westphalia (NRW) within a "cordon" along the border of The Netherlands and from a control area in northern Germany (SH=Schleswig-Holstein). (+) indicates foot-and-mouth disease outbreaks (livestock) in The Netherlands in 2001.

could likely be infected with and could potentially spread FMDV (Boardman et al., 2001).

During 2001 (21 March to 22 April), a FMD outbreak occurred in The Netherlands. The objective of this study was to determine, through serologic testing, whether free-ranging roe deer in adjacent areas of Germany were exposed.

In collaboration with local hunters, 223 coagulated blood samples were collected from free-ranging roe deer between October 2001 and October 2002. Blood was collected from the heart immediately after death and was sent to the Institute for Zoo and Wildlife Research, Berlin, Germany, or to the Friedrich-Loeffler-Institute, Riems, Germany, within 1–10 days. Samples originated from Lower Saxony (n=43; 51°17′–53°53′N, 6°40′–11°35′E) and North-Rhine Westphalia (n=108; 50°19′–52°19′N, 5°52′–9°28′E) and were collected within a cordon extending approximately 20 km from the border of The Netherlands (Fig. 1). Additional samples were collected from selected hunting grounds in Schleswig-Holstein (n=72; 54°30′–54°50′N, 9°20′–9°40′E) designated as control areas. Samples were collected from 48 fawns, 90 yearlings, and 82 adults; age information was unavailable for three deer. Whole blood was centrifuged, and serum was removed and frozen at −20 C. Before analysis, sera were inactivated at 56 C for 30 min. Sera were tested for antibodies against FMDV serotype O Manisa with two enzyme-linked immunosorbent assays (ELISAs) and virus neutralization.

The ELISA was performed according to Hamblin et al. (1986a, b) as a liquid phase blocking ELISA (LPBE). Briefly, microtiter plates (Maxisorp, Nunc GmbH, Wiesbaden, Germany) were coated with rabbit anti-FMDV hyperimmune serum and

stored at −20 C (ELISA plates). Duplicate twofold series of each test serum (50 μl/well) were prepared in U-bottomed plates (PS-microplate, Greiner GmbH, Frickenhausen, German) in phosphate-buffered saline supplemented with 0.1% Tween 20 (PBST), starting at a dilution of 1:20 (carrier plates). Foot-and-mouth disease virus antigen (50 μl) was added to each well, resulting in a final dilution of 1:40. Carrier plates were incubated at 4 C overnight. After thawing and washing, the antibody-coated ELISA plates received 50 μl/well of the serum/antigen mixtures from the carrier plates. After incubation at 37 C for 60 min and a washing step, the wells were further incubated with 50 μl of homologous guinea pig anti-FMDV hyperimmune serum, preblocked with normal rabbit serum, at 37 C for 60 min. After washing, 50 μl of goat anti-guinea pig immunoglobulin G (IgG) conjugated to horseradish peroxidase (Dianova, Hamburg, Germany), preblocked with normal rabbit serum, was added to each well, and the plates were incubated at 37 C for 30 min. After another washing procedure, the bound enzyme reacted for 10 min with the chromogen (ortho-phenylenediamine supplemented with perhydrol; Sigma Chemie GmbH, Deisenhofen, Germany). The reaction was stopped with H_2SO_4, and the results were read at 492 nm on an ELISA-Reader (Digiscan, Asys Hitech GmbH, Eugendorf, Austria). Controls in each test included eight wells containing only antigen—but no serum—to generate the 100% value, four wells containing antigen mixed with a weak positive bovine serum (Friedrich-Loeffler-Institute, Riems, Germany), and four wells without antigen (blank wells). The blank wells served as quality control, which should not exceed an optical density (OD) of 0.150. The weak positive control wells served as quality control for the sensitivity of the test. Antibody titers were expressed as the final dilution of serum giving 50% of the OD of the 100% value. Titers of ≥40 were considered positive.

For the second ELISA, this procedure was modified according to MacKay et al. (2001) to a solid phase competition ELISA (SPCE). Briefly, antibody-coated ELISA plates (see LPBE) were washed, and 50 μl of FMDV antigen was added to each well except the blank control wells. Plates were incubated for 60 min at 37 C. Meanwhile, 25 μl of each test serum was mixed with 100 μl of homologous guinea pig anti-FMDV hyperimmune serum diluted in SPCE buffer (PBST supplemented with 10% fetal bovine serum and 2% normal rabbit serum) in U-bottomed plates, resulting in a dilution of 1:5. After washing, each well of the ELISA plates received 50 μl of the test serum/guinea pig anti-FMDV hyperimmune serum mixtures prepared in the U-bottomed plates. After an incubation period of 60 min at 37 C and a washing procedure, 50 μl of goat anti-guinea pig IgG conjugated to horseradish peroxidase and preblocked with normal rabbit serum was added to each well. After another incubation period at 37 C for 30 min, the above-described procedure for the LPBE followed. Controls in each test included eight wells containing no serum to generate the 100% value, four wells containing a weak positive bovine serum, and four wells that had received no antigen (blank wells). The blank wells served as quality control, which should not exceed an OD of 0.150. Antibody titers were expressed as the last dilution of serum, giving ≤70% of the OD 100% value. Titers ≥5 were considered positive.

All LPBE-positive sera were tested with a virus neutralization test (VNT) modified according to Golding et al. (1976). Briefly, all tests were performed with baby hamster kidney cells (BHK21-CT) and minimal essential medium (MEM; Gibco BRL Life Technologies Inc., Gaithersburg, Maryland, USA) in 96-well microtiter plates (Nunclon Microwell plate, Nunc GmbH, Wiesbaden, Germany). Foot-and-mouth disease virus strain O Manisa was used for the detection of FMDV-specific antibodies. To reduce the variability of se-

rum titers resulting from deviations of the actual virus titer from the target virus titer of 100 median tissue culture infective doses ($TCID_{50}$/50 μl), each test serum was incubated with three different challenge virus dilutions (CVDs). While the aim for CVD 2 was 100 $TCID_{50}$/50 μl, the challenge virus dilutions CVD 1 and CVD 3 contained 400% and 25% of the amount of virus in CVD 2, respectively. On the basis of a virus titration included in each test, the CVD that gave an actual virus titer close to the target virus titer was determined and used to calculate the serum titers. The sera were titrated starting at 1:4 for the test sera and 1:40 for the bovine control serum. Serum dilutions (50 μl) were incubated with 50 μl of the CVDs for 2 hr at 37 C in an atmosphere of 5% CO_2. Subsequently, 100 μl of cell suspension (2×10^6 cells/ml) were seeded into each well. Plates were incubated in 5% CO_2 at 37 C. Four days later, the cell cultures were evaluated microscopically for the presence of cytopathic effects. Controls included a positive bovine control serum of known titer, a cell control, and a virus titration in fourfold steps with eight replicates per step, starting with CVD 1. The test was considered valid when the titer of the positive control serum was within twofold of its expected titer and the amount of virus in 50 μl was within the range of 31.6–316.0 $TCID_{50}$. Serum titers were expressed as the reciprocal of the highest final dilution of serum that resulted in 50% of the wells being protected from a cytopathic effect. Titers ≥8 were considered positive.

The McNemar test was used to compare results between LPBE and SPCE, LPBE and VNT, and SPCE and VNT. The significance level was set to $\alpha=0.05$ and adjusted for multiple testing with the Bonferroni correction (Sachs, 1997).

With LPBE, 12 of 223 (5.4%) sera tested positive for antibodies against FMDV. These samples originated from North-Rhine Westphalia ($n=6$), Lower Saxony ($n=2$), and Schleswig-Holstein (control area, $n=4$). With SPCE, 26 of 223 (11.7%) sera tested positive (North-Rhine Westphalia, $n=7$; Lower Saxony, $n=7$; Schleswig-Holstein, $n=12$). Eleven of 12 LPBE-positive serum samples were positive in the SPCE. However, all LPBE-positive sera tested negative by VNT. Significant differences were detected when comparing results between LPBE and SPCE ($P=0.0005$), LPBE and VNT ($P=0.0005$), and SPCE and VNT ($P<0.0001$).

In this study, confirmed FMD seropositive results from roe deer were not detected. These results suggest that FMDV was not transmitted to free-ranging roe deer in the investigation area during the outbreak in 2001. This is consistent with similar investigations from the UK and The Netherlands in 2001. The FMD epidemic in the UK started in February 2001 and was caused by FMDV serotype O strain PanAsia (Knowles et al., 2001; Davies, 2002). During this epidemic, several "wild deer" (species not mentioned) with typical signs of FMD were reported (Davies, 2002; Griot and Giacometti, 2002). However, all test results from 107 diagnostic samples and 474 serum samples were negative (D. Paton, pers. comm.). In The Netherlands, the first outbreak of FMD was confirmed on 21 March 2001 on a dairy farm, and the last case was reported on 22 April 2001 (Bouma et al., 2003). During this outbreak, lameness and abnormal gait were reported in roe deer from a nature reserve in the center of the epidemic area, but infection with FMD was not confirmed (Sutmöller, 2001). Antibodies to FMD infection were not detected from blood samples from "captive deer" (species not mentioned), which were culled on eight deer farms in the vicinity of infected livestock (Elbers et al., 2003). Furthermore, 140 blood samples collected from culled free-ranging roe deer between 25 June and 26 October 2001 in areas in proximity to infected premises all tested negative (Elbers et al., 2003).

A possible role of wildlife in the maintenance and transmission of FMDV dur-

ing an epidemic in livestock in Germany in 1935 has been discussed (Bartels and Claassen, 1936), and large populations of wild deer that might come into close contact with domestic livestock exist in many rural areas. However, virus transmission from domestic livestock to wild deer has only been reported during the first four decades of the 20th century in Europe (Bartels and Claassen, 1936; Cohrs and Weber-Springe, 1939; Sallinger, 1939; Stroh, 1939). During FMD outbreaks in the last six decades, "spillover" from infected livestock to free-ranging deer or "spillback" has not been reported (Hess, 1967; Kubin, 1972; Anonymous, 1982). Between 1961 and 1973, deer from the UK were examined for antibodies against FMDV serotypes O, A, and C, but seropositive animals were not detected (Lawman et al., 1978). To date, there is no evidence that wild deer have played an important role in the epidemiology of epidemic FMD in western Europe (Nevermann, 1914; Bartels and Claassen, 1936; Waldmann and Hirschfelder, 1938; Gibbs et al., 1975; Wilesmith, 2001).

For the diagnosis of FMD in roe deer, procedures similar to those described for livestock can be applied. LPBE and VNT are the prescribed tests for the detection of FMDV-specific antibodies (Anonymous, 2000). Presently, VNT is recommended as the definitive "gold standard" for the final assessment of results (Alexandersen et al., 2003). Antibodies against FMDV detected in the serum of cattle recovering from FMD are of the early and the late types (Brown and Graves, 1959). In cattle, early antibodies (largely IgM) can usually be detected at 4 days postinfection (PI) and antibody titer peaks at approximately 10–14 days PI. These antibodies decline to low levels within 30–40 days PI (Brown et al., 1964; Cowan, 1966). Late-appearing antibodies (IgG) are detected 10–14 days PI and persist for several months (Cowan and Trautman, 1965; Cowan, 1966), and both types of antibodies are neutralizing (Cowan and Trautman, 1965). The antibody response can be detected for many months and even years after infection (Alexandersen et al., 2003), but some species-related variation can occur. Cattle infected with FMDV serotype O had high levels of circulating and neutralizing antibodies up to 18 mo PI (Gomes et al., 1972). African buffalo (*Syncerus caffer*) and kudu (*Tragelaphus strepsiceros*) experimentally infected with FMDV serotype SAT 2 had neutralizing antibody titers similar to those in cattle, and antibody response persisted throughout the experiment (300 days). However, antibodies in impala (*Aepyceros melampus*) did not persist at a significant level beyond 300 days (Hedger et al., 1972).

Liquid phase blocking ELISA is routinely used for detection of FMDV-specific antibodies. Although the reported sensitivity of LPBE is approximately 100% and its specificity 95%, these values can vary, and in some cattle populations, especially if under stress, the percentage of animals giving false-positive results can reach 18% (MacKay et al., 2001; Alexandersen et al., 2003). In this study, 5.4% of the sera tested positive by LPBE. Because positive results could not be confirmed by VNT, they were regarded as false-positive. In addition, 11.7% of the sera showed positive results by SPCE. They were considered false-positive because they could not be confirmed with LPBE and VNT. Preliminary specificity trials with German cattle sera (Haas, unpubl. data) appear to confirm the findings of MacKay et al. (2001), who reported a high sensitivity and specificity (99.8%) of the SPCE for this species. Thus, either roe deer sera in SPCE was problematic or the hemolysis of roe deer sera had a negative effect on specificity. This is possible because a 1:5 serum dilution was used for the SPCE.

Although no seropositive reactors were found in this study, potential contact of roe deer with FMDV in the investigation area cannot be excluded. However, considering the contagiousness of the virus, which usually results in a high rate of infection, this

possibility seems unlikely. Nevertheless, the following aspects should be considered in the interpretation of these serologic data. First, it could be possible that antibodies against FMDV in roe deer are only detectable for a few months and were no longer present at the time the samples were collected. Second, the number of possibly exposed animals was reduced because fawns, which represented 25.8% of Lower Saxony and North-Rhine Westphalia samples, were not born until after the outbreak in The Netherlands (March and April 2001). Finally, it might be possible that individual roe deer became infected but died before the sampling period.

Our results suggest that FMDV was not transmitted to free-ranging roe deer in Germany during the FMD outbreak in The Netherlands in 2001. In addition, the SPCE in its current form does not seem to be an appropriate method for detecting antibodies against FMDV in hunted roe deer because of problems associated with specificity.

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