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Source: Journal of Wildlife Diseases, 32(2) : 348-353

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-32.2.348

Journal of Wildlife Diseases, 32(2), 1996, pp. 348–353

Seroprevalence of Orthopox Virus Specific Antibodies in Red Foxes (*Vulpes vulpes*) in the Federal State Brandenburg, Germany

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ABSTRACT: The prevalence of orthopox virus (OPV)-specific antibodies in 1,040 red foxes (*Vulpes vulpes*) was evaluated on a large scale in the German Federal State Brandenburg. Serum samples were selected from 809 communities within the study area from January 1991 to September 1994 by simple random sampling. Screening was carried out by an indirect enzyme-linked immunosorbent assay (ELISA). Orthopox virus-specific antibodies were found in 162 (16%) of the 1,040 fox sera. Furthermore 154 (15%) sera were considered suspect positive. The specificity of the antibodies detected in ELISA-positive and suspect positive sera was confirmed by Western blotting. Presence of OPV-antibodies occured in 291 communities. No correlation of OPV-antibodies findings to latitude or characteristic topographical and ecological peculiarities of the study area was found. Although the causative agent is still unknown we believe that orthopox viruses probably have a ubiquitous presence among red foxes.

Keywords: Red fox, *Vulpes vulpes*, Orthopox virus, ELISA, seroprevalence, seroepidemiology, spatial distribution.

In 1980, smallpox (variola) was declared as eradicated from the human population (World Health Organization, 1980). However, other members of the genus orthopox virus (OPV) such as cowpox, camelpox, mousepox, or monkeypox are important pathogens for humans and other animals. Since 1974, infections of zoo animals (Marennikova et al., 1977; Pilaski et al., 1986) and cats (Zimmer et al., 1990; Mahnel, 1991) have been described in Europe. Using molecular techniques, the causative agents were identified as cowpox virus (Naidoo et al., 1992). Bennett et al. (1989) postulated that a reservoir exists in small rodents, and that humans, zoo animals, or cats are the final host species. This hypothesis is supported by several serological surveys in cats (Bennett et al., 1989) and small field rodents such as the long tailed field mouse (*Apodemus sylvaticus*) and the field vole (*Microtus agrestis*) (Kaplan et al., 1980), as well as by virus isolation from white rats in the Moscow Zoo, Russia (Marennikova and Shelukina, 1976; Marennikova et al., 1984) and the wild big gerbil (*Rhombomys opimus*) in Turkmenia (Marennikova et al., 1978). However, the epizootiology of the disease still is poorly understood. Due to its special nutritional behavior, the red fox (*Vulpes vulpes*) might be a member of this infectious chain as its preferred food consists mainly of small rodents (Matejka et al., 1977; Lloyd, 1980). Evidence for participation of the red fox in the infection cycle was obtained by Henning et al. (1995), who detected OPV-specific antibodies in 6.5% of the fox serum samples in a small area in northern Germany. This was the first known report of an immune response to OPV in free-ranging red foxes. Our objective was to test these preliminary results in the larger neighboring regions. Furthermore, it was also the aim of this study to obtain information concerning a possible relationship of the spatial distribution of serologically OPV-positive findings and ecological and topographical peculiarities of the study area as well.

A retrospective seroepizootiological study was carried out in the German Federal State, Brandenburg (51°28′ to 53°24′ N, 12°17′ to 14°45′E), an area of 23,924

km^2 east of the study area described by Henning et al. (1995). Samples were obtained either from a wildlife disease monitoring project or from follow-up investigations of oral fox vaccination campaigns against rabies originating from hunting areas in Brandenburg. Carcasses of red foxes, which had been shot, trapped, or found dead between January 1991 and September 1994 were collected by hunters and local veterinarians. Subsequently, they were sent to one of the three State Veterinary Laboratories in Brandenburg (Potsdam, Cottbus, and Frankfurt/Oder) located within the study area. Conveyance of the carcasses by courier took between 1 and 5 days. Prior to investigation all serum samples were centrifuged and stored at −30 C in a central serum bank for wildlife established at the Federal Research Center for Virus Diseases of Animals, Wusterhausen, Germany. Their place and time of origin were recorded.

The minimum sample size required was determined using the Cannon and Roe (1982) statistical procedure for populations with unknown densities. Using the software EPI-INFO Version 5.00—(April 1990, Public Domain Software for Epidemiology and Disease Surveillance, Centers for Disease Control, Epidemiology Program Office, Atlanta, Georgia, USA—Statcalc Epi Calculator) the calculation was based on the prevalence found previously by Henning et al. (1995). As a consequence, the lowest acceptable hypothetical prevalence (=95% confidence interval) was set as 6.5% ± 1.5%. The significance level was set at $\alpha = 0.05$.

Between January 1991 and September 1994, 2,871 fox sera were stored in the wildlife serum bank, representing 809 of 1,496 communities within the study area. According to the sample calculation a minimum of 1,040 fox serum samples were required. The selection of samples was aligned to guarantee a nearly equal geographical distribution of the calculated sample size over the study area. Thus, all communities within the study area from which fox sera were available were involved in the serological screening.

Initially, one serum sample was indiscriminantly selected from each of these 809 communities (292 of these had only one serum sample). To reach the required sample size a second serum sample was indiscriminantly selected from 231 of the 517 communities represented by more than one serum sample. Using desktop mapping software (cartographic software "RegioGraph", Version 1.2 a, March 1993, Macon Markt und Konzept, Waghäusel, Germany), these 231 communities were found to be nearly equally distributed within the study area. This cartographic software was also used to plot the results of the serological screening on maps.

Screening of fox serum samples for OPV-specific antibodies was performed at a dilution of 1:2 in a blocking-enzyme linked immunosorbent assay (ELISA) as described by Henning et al. (1995), using a genus-specific monoclonal antibody (mAb) anti-KR2-3D11 (Czerny and Mahnel, 1990). Serum of a farm fox free of antibodies directed against OPV based on a plaque-reduction test (Mayr et al., 1977), ELISA, and immunoblotting (Towbin et al., 1979) served as a negative control. A polyclonal hyperimmune serum against purified vaccinia virus strain Elstree was produced in rabbits and used as a positive control. According to Henning et al. (1995), a reduction of the optical density (OD) of equal or more than 70% was regarded as positive. Sera with a reduction of the OD between 50% and <70% were suspected to contain OPV-antibodies. A selection of these samples further was evaluated by Western blot analysis for specificity (Towbin et al., 1979). The 95% ($\alpha = 0.05$) confidence interval limits (CI) for the estimation of the true seroprevalence within the fox population were calculated according to the method of Willer (1982).

On screening fox sera in the blocking-ELISA, 162 (16%) sera caused a reduction of the OD of ≥70%. Furthermore, 154 ad-

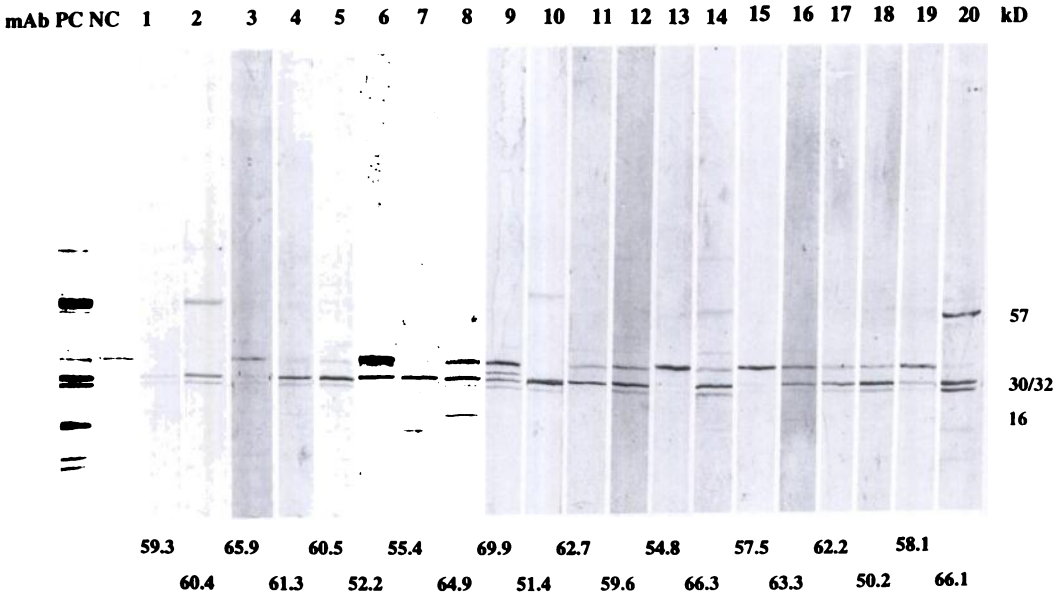

FIGURE 1. Results of Western blot immunoassay serologically confirming specificity of OPV-antibodies of ELISA-suspect fox sera: mAb = monoclonal antibody anti-KR2-3D11, PC = positive control (rabbit hyperimmune serum), NC = negative control (farm fox). Lanes 1 to 20 are the reactions of ELISA-suspect fox sera. Values below are the percent reduction of enzyme-linked immunosorbent assay-optical density (ELISA-OD). Lanes 3, 11, 13, and 15 were considered negative. To a lesser degree proteins of the 75 kD, 16 kD, and 14 kD were detected (lanes 2, 6, 12, 14, 18, and 20).

ditional sera had a reduction between 50% and <70% and were considered suspect-positive. Of these, a selection of 40 sera were further evaluated by Western blot analysis. Thirty-five (85%) of these 40 sera bound intensively with the specific OPV-32/30 kilodalton (kD) proteins, which represent the epitope reacting with the mAb anti-KR2-3D11. Fox sera also bound to the 57 kD protein, and sometimes to a lesser degree to the 14 and 16 kD proteins of OPV (Fig. 1). These reactions are affected by slight background coloring, which was due to the poor quality of some of the serum samples.

Sera with a OD-reduction of ≥70% and sera with a reduction between 70% and 50% could be attributed to 151 and 140 communities, respectively (Fig. 2). There was no obvious relationship between antibody prevalence and topographical or ecological characteristics of these study areas.

Our results are in agreement with and extend the findings of Henning et al. (1995); they observed a prevalence of 6.5% OPV-positive fox sera based on a reduction of the OD of more than 70%. This 6.5% figure was regarded to be a minimal figure. Based on the same cut-off, 162 (16%) of 1,040 fox sera in our study were seropositive in the blocking-ELISA; we believe this represents a true seroprevalence within the 95% CI-limits of 13% and 17%. Similarly, C.-P. Czerny (unpubl.) found 10 to 15% seropositive foxes in regions of Baden-Württemberg, Germany. However, the prevalence of OPV-specific antibodies in red foxes might be even higher than 16%. Henning et al. (1995) found that all sera whith an OD reduction of ≥70% also were positive for OPV-antibodies in Western blot tests. We found, that 35 (85%) of 40 tested sera leading to a reduction of the OD between 50% and <70% also had specific OPV-32/30 kD bands (Fig. 1), which is the immunodominant protein for the genus orthopoxvirus

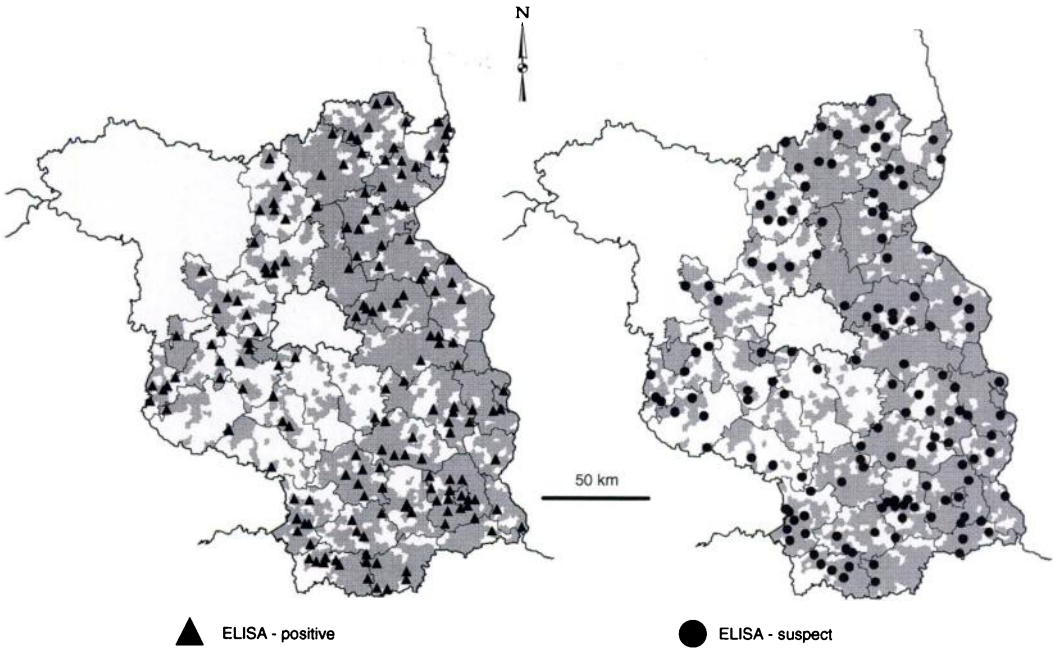

FIGURE 2. Distribution of communities with OPV-specific antibody reactions in Brandenburg as determined by serological testing of red fox sera in the blocking enzyme-linked immunosorbent assay (ELISA) sampled from January 1991 to September 1994; ELISA-positive = reduction of the ELISA-optical density ≥70%, ELISA-suspect = reduction of the ELISA-optical density between >50% and <70%.

(Czerny and Mahnel, 1990; Maa et al., 1990). Based on this finding we speculate that most (85%) of the 154 sera considered ELISA-suspect probably have OPV-specific antibodies, too. Thus, we predict that the true seroprevalence among red foxes in our study area may be 28% or higher.

The spatial distribution of fox sera having OPV-specific antibodies was related to 291 communities. Henning et al. (1995) suggested a relationship between clusters of positive sera and topographical conditions such as rivers, brooks, and lakes. The sample size of the present study is representative for the study area but due to the sample selection it is not possible to make inferences on a community basis. In our study, however, possible natural foci could not be identified by desktop mapping. Based on the geographical pattern of communities with OPV-positive fox sera (Fig. 2), we speculate that OPV-antibodies in red foxes may occur throughout Brandenburg independent of topographical and ecological characteristics of the study area. Since a further subdivision of communities was not possible, however, the existence of such foci at a lower level can not be ruled out completely.

The origin of the OPV-antibodies in red foxes is unknown. Although, orthopox virus infections are widespread in animals (Baxby, 1977) epizootiological interactions are poorly understood. At present four agents must be considered as possibly evoking OPV-specific antibodies in free-ranging red foxes: a mousepox virus (Marennikova et al., 1984; Mahnel et al., 1993), a cowpox virus, a hypothetical 'foxpox virus' (Henning et al., 1995) or a vaccinia virus.

On the one hand, the high seroprevalence in red foxes may result from an enzootic occurrence of OPV in red foxes comparable to latent OPV infections in raccoons (*Procyon lotor*) as known from North America (Esposito and Knight, 1985; Fenner et al., 1989). However, Hen-

ning et al. (1995) speculated that the OPV-antibodies in red foxes may be evoked more likely by an infection with a cowpox-like virus than by a hypothetical 'fox pox' virus. Similarly, cowpox viruses also were detected in cats (Mahnel, 1991). However, in cat populations a horizontal transmission is not supposed due to the low infectivity of the isolates (Bennet et al., 1989).

On the other hand, this high antibody prevalence in red foxes is consistent with the hypothesis that the population of small rodents is infected and may represent the true reservoir (Bennett et al., 1989). This is supported by several virus isolations from *Rhombomys opimus* in Turkmenia, Asia (Marennikova et al., 1978). Recently, an orthopox virus (volepox) was also identified in North America in *Microtus californicus* (Regnery, 1987). Marennikova et al. (1984) also demonstrated OPV-antibodies in *Mus musculus* (0.25%) and *Rhombomys opimus* (17%) captured from West Russia and Poland. Ectromelia virus (mousepox) causes acute or chronic diseases especially in laboratory mice but seems to be more latent in wild mice (Fenner et al., 1989). Interestingly, an enzootic occurrence of mousepox virus was also observed in several furbearing animal farms in the Czech Republic, where this ectromelia-like virus caused congenital ectromelia infections in puppies of silver foxes (*Vulpes fulva argentata*) and minks (*Mustela lutreola*) (Mahnel et al., 1993). From our data, we cannot clearly identify rodents as the orthopox reservoir for infection in free-ranging red foxes but there is nothing known about other potential sources of OPV.

However, the induction of OPV-specific antibodies caused by recombinant vaccinia virus vaccines against rabies (Blancou et al., 1986; Pastoret et al., 1988, 1992) can be ruled out completely, because in Germany only modified life rabies virus vaccines have been used so far for oral immunization of foxes against rabies (Schneider and Cox, 1983; Stöhr et al., 1994). Boulanger et al. (1995) outlined the potential risk of a recombination of vaccinia recombinant viruses with enzootic occurring orthopox viruses. Based on experimental studies they believe it is very low under natural conditions. To identify those viruses in our study sites, we have attempted to isolate the virus from red foxes, small rodents, and other potential sources in these areas.

This research was supported in part by the Government of the Federal State of Brandenburg, Ministry of Food, Agriculture and Forestry. We acknowledge the State Veterinary Laboratories of Frankfurt/Oder, Potsdam, and Cottbus for assistance in collection of field samples and for their cooperation during the last years. Technical support for the serological assays and for the processing of field data was kindly provided by Susann Klähn, Ronald Schröder and Detlef Klöß.

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Received for publication 21 February 1995.