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## An Indirect Immunofluorescent Test for Detection of Rabies Virus Antibodies in Foxes

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**ABSTRACT:** The blood-containing fluids in the thoracic cavity or blood from the heart from 177 red foxes (*Vulpes vulpes*) in Slovenia were evaluated for rabies antibodies by rapid fluorescent focus inhibition test (RFFIT) and an adapted indirect immunofluorescent test (IIF) in 1994. We evaluated the usefulness of anti-dog fluorescein-isothiocyanate (FITC) conjugate instead of anti-fox FITC conjugate in detection of antibodies against rabies virus in fox sera. In the RFFIT test, 92 (52%) of the fox samples were positive and 70 (40%) samples were negative for rabies antibodies; 15 (8.5%) samples were not suitable for examination in this test. In the IIF test, 98 (55%) fox samples were positive and 79 (45%) sera were negative. The IIF test was suitable for the rapid detection of antibodies against rabies virus in foxes, as often required for vaccine efficacy trials.

**Key words:** rabies, serology, foxes, *Vulpes vulpes*.

Rabies virus is the member of the genus *Lyssavirus* in the Rhabdoviridae family (Mathews, 1979). This fatal neurotropic virus infects a wide range of warm-blooded animals (Charlton, 1987). Red foxes (*Vulpes vulpes*) are the main vectors and reservoirs of rabies in central Europe (King and Turner, 1993). In recent years, rabies control was based on population reduction of foxes (Seidler et al., 1982). Oral vaccination of foxes is another method used to eradicate rabies (Blancou et al., 1988). Animals can be immunized by inoculating live attenuated rabies virus Street Alabama Dufferin (SAD), strain SAD-Bern directly in the mouth cavity. The red foxes developed protective antibodies (Baer et al., 1971).

Several methods are used for detection of antibodies against rabies virus in seroepizootiological studies after oral vaccination of foxes: the rapid fluorescent focus inhibition test (RFFIT) (Smith et al., 1973), indirect immunofluorescent test (IIF) (Smith et al., 1973), and the enzyme-

linked immunosorbent assay (ELISA) (Mannen et al., 1987). Esterhuysen et al. (1994) reported a liquid-phase blocking ELISA test for detection of antibodies to rabies sera in various animal species.

Evaluation of oral immunization of foxes in field trials involve detection of antibodies against rabies virus in the blood-containing fluids in the thoracic cavity or in heart blood from carcasses of red foxes, and by the detection of a marker such as tetracycline in the teeth and bones of foxes (Schneider, 1988). Postvaccinal immunity in rabies can be assessed directly by challenge experiments in foxes or by detecting the specific antibodies against rabies virus. The neutralizing antibodies in rabies infection have been regarded as an important factor in postexposure protection (Habel and Koprowski, 1955). Jayakumar and Ramadass (1991) found that dogs having specific immunoglobulin G (IgG) antibodies against rabies virus did not die from rabies after experimental infection with rabies virus, but the dogs with only specific IgM antibodies died after infection.

In Slovenia the first rabies case in foxes was diagnosed in 1973 near the Hungarian border. The disease spread south-west and covered the country within a few years (Bidovec et al., 1993). In 1995, 3,787 samples were tested for the presence of rabies in Slovenia, with 1089 positive samples. Of 2,726 red foxes evaluated for rabies in 1995, 996 (37%) were positive for rabies.

In 1988, a vaccination campaign in Slovenia (45°30' to 47°20'N, 13°30' to 16°39'E) was conducted. The vaccination campaign followed the Bavarian model (Schneider and Cox, 1983) and was carried out zone by zone, from the western border to the east. The oral vaccination in Slovenia was not performed in a continuous program because of budgetary constraints.

The 177 samples of blood-containing liquid in the thoracic cavity or heart blood were collected from foxes killed by hunters. Collection of samples was started 1 mo after bait distribution in April 1994. All samples were tested by the RFFIT test (Smith et al., 1973) at the World Health Organization Reference Center, Tübingen, Germany, and by the IIF test in our laboratory. In this study, the IIF (Johnson and Richard, 1992) test for the detection and titration of antibodies to rabies in blood serum samples of foxes was used. The commercial anti-dog FITC conjugate (Sigma Chemical Company, St Louis, Missouri, USA) was used instead of anti-fox FITC conjugate. Results were compared with RFFIT test.

The IIF test was performed like that the SAD-B19 virus strain was propagated in Baby Hamster Kidney (BHK 21) cell line; the cells were supplied by Dr. Lathar Günter Schneider, the Federal Research Center for Virus Diseases of Animals, Tübingen, Germany. Cell cultures were grown in minimal essential medium (MeM) (Gibco, Life Technologies Ltd, Paisley, United Kingdom) supplemented with 10% sheep serum and 0.1% garamycin (Lek, družba z omejeno odgovornostjo, Ljubljana, Slovenia).

The 10 ml of BHK<sub>21</sub> cell suspension ( $1 \times 10^5$  cells/ml medium) were infected with 0.2 ml SAD B19 strain rabies virus immediately after trypsinization (Grist et al., 1981); the virus titer was  $1.5 \times 10^4$  tissue culture infective doses (TCID<sub>50</sub>) per ml. The infected cells were incubated at 37 C for 2 days, trypsinized (Harald and Emmons, 1992), and mixed with uninfected cells in a 1:1 ratio. The pool of cells ( $8 \times 10^4$  cells/ml) were plated onto slides and incubated a further 24 hr at 37 C and in 5% CO<sub>2</sub>. The slides then were fixed with acetone, air-dried and stored at -20 C. In microplates, we prepared two-fold dilutions, from 1:2 to 1:1,048, of examined fox blood samples in phosphate-buffer (PBS), pH 7.2. The inoculum of 0.025 ml of each blood sample dilution was added onto ap-

propriate spots. The slides were incubated for 60 min, washed for 20 min in PBS, rinsed with distilled water and air-dried. Anti-dog IgG conjugate (Sigma Chemical Company, St. Louis, Missouri, USA) at a 1:40 dilution was added to each spot in a volume 0.025 ml. The slides were incubated for 60 min at 37 C, and washed twice in PBS; we then added glycerin and covered them with coverslips. The slides were examined under an Opton fluorescent microscope (Opton Feinttechnik GmHb, Oberkochen, Germany). In positive cases, the typical granular intracytoplasmic inclusions were observed. The antibody titer was the highest serum dilution giving positive fluorescent staining in cells.

Blood samples from 177 foxes were tested by the IIF and RFFIT test. Ninety-two (52%) fox blood samples were positive, 70 (40%) samples were negative and 15 (8%) samples had cytotoxic activity in the RFFIT test; titers varied from less than 1:60 to 1:1,620. Ninety-eight (55%) samples were positive and 79 (45%) samples were negative in IIF test; titers varied from less than 1:2 (negative) to 1:1,024. All samples were suitable for examination. Two sera were RFFIT-positive, but IIF-negative.

The neutralization tests and IIF tests do not measure an identical spectrum of antibodies. The neutralization of serum samples, as measured by the RFFIT test, is due to the presence of virus-specific neutralizing antibodies directed against the surface glycoprotein, but in the IIF test antibodies are directed against the nucleocapsid proteins (Schneider et al., 1973; Campbell and Barton, 1988). The presence of specific antibodies to rabies virus with RFFIT test or IIF test was evidence, that the foxes encountered rabies antigen in the past. A poor correlation between the IIF test with the mouse-neutralization test has been reported (Grandien, 1977; Barton and Campbell, 1988); in contrast, good correlations between the IIF test and RFFIT in recognizing positive and negative reactors have occurred (Hill, 1990).

The samples of blood-containing liquid

in the thoracic cavity and heart blood collected from foxes killed by hunters were usually toxic and contaminated with bacteria. These samples in low dilution were not suitable for the RFFIT test, because the nonspecific toxicity for the cells was observed (Schneider and Cox, 1983). The IIF test described here is simple and sensitive and correlates well with results of the RFFIT test; it also has the advantage that carnivore sera can be tested and the results can be produced in a single work day, if slides were prepared and stored earlier. From these results, we conclude that the IIF test is a simple method for seroepizootiological studies after oral vaccination of foxes.

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