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Serologic Evidence of Bovine Viral Diarrhea Virus in Free-ranging Rabbits from Germany

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ABSTRACT: From 1993 to 1995, 100 blood samples and 97 spleen samples of free-ranging rabbits (*Oryctolagus cuniculus*) from northern Germany were tested for prevalence of different bovine viral diarrhea virus (BVDV) antibodies and BVDV antigen, respectively. Forty sera (40%) were positive for antibodies to one or more of three cytopathogenic BVDV strains (NADL, Grub 313/83, and roe deer isolate SH9/11). Microneutralization test (NT) titers ranged from 5 to 13. Moreover, 13 of 35 NT positive sera also were positive by enzyme-linked immunosorbent assay for detection of anti-BVDV antibodies. Therefore, we assume that the rabbits either had passively acquired antibodies or had experienced natural infection with BVDV. Furthermore, 97 spleen samples were used for attempts to isolate BVDV and tested by reverse transcription polymerase chain reaction for viral nucleic acid using various protocols. Bovine viral diarrhea virus was not identified in these samples.

Key words: Bovine viral diarrhea virus, *Oryctolagus cuniculus*, serologic survey, polymerase chain reaction.

Bovine viral diarrhea virus (BVDV) belongs to the genus *Pestivirus* within the Family Flaviviridae (Horzinek, 1990). Bovine virus diarrhea (BVD) is a generalized viral infection affecting a broad range of hosts including domestic rabbits (Baker et al., 1954; Nettleton, 1990). Until now, there was no indication that free-ranging rabbits (*Oryctolagus cuniculus*) may be carriers of BVDV. Only experimental BVDV infections have been reported in rabbits (Baker et al., 1954; Fernelius et al., 1969; Saurat et al., 1973). The intravenously inoculated domestic rabbits showed no signs of illness and necropsy, none of the rabbits had lesions of BVD infection. However, calves inoculated with material from these rabbits developed typical signs of BVD (Baker et al., 1954).

The natural mode of BVDV infection in free-ranging rabbits and the question of whether they could serve as a reservoir is not clear yet (Frölich, 1995). Direct contact is unlikely to occur between cervids or domestic livestock and free-ranging rabbits. However, indirect transmission has been reported (Meyling et al., 1990).

Serologic surveys for BVD have been conducted in a variety of wild species on many continents (Nettleton, 1990). According to Frölich (1995), seven (7%) of 94 free-ranging cervids in one investigation area had antibodies against BVDV. Possibly there is a natural focus of infection for free-ranging cervids. Cattle may be of minor importance in the infection process (Frölich, 1995); wild boars (*Sus scrofa*) (Dahle et al., 1993) and rabbits also may be potential carriers of BVDV. Our objective was to determine if free-ranging rabbits in Germany were naturally infected by BVDV and, therefore, could be involved in the epidemiology of this virus.

Rabbits were culled by local hunters, who had been supplied with blood and spleen tubes. Blood and spleen were collected immediately after death and sent to our laboratory (Institute for Zoo Biology and Wildlife Research, Berlin, Germany) within 1 to 4 days. The samples originated from hunting areas in the northern part of Schleswig-Holstein, Germany (54°30′N to 54°50′N, 9°00′E to 9°30′E), where rabbits and deer, but not wild boars, are sympatric (Wiese, 1993). A high density of cattle (>2.6 animals per ha of cultivated area) was present (Anonymous, 1987). One hundred usable blood samples and 97 usable spleen samples were collected between August 1993 and July 1995. After centrifugation, serum samples were heated to 56 C for 30 min and stored at −20 C. Spleen samples were stored at −70 C.

A microneutralization test (NT) as described by Frey and Liess (1971) and Frölich (1995) was applied for the detection of BVDV antibodies. Three cytopathogenic BVDV strains were used for the NT: (1) SH9/11, recently isolated from a free-ranging female roe deer (Frölich and Hofmann 1995), (2) Grub 313/83 (kindly provided by G. Wizigmann, Zentralinstitut für Tiergesundheit, Grub, Germany), and (3) NADL. Antibody titers were calculated according to Spearman and Kärber (1985). Titers >4 were considered indicative of natural exposure and will be referred to as positive (Malmquist, 1968). Sera were considered positive if they had antibodies to one or more of the three BVD viruses. All samples were tested twice in this assay and the mean titer was calculated. Control sera were available from rabbits kept specific pathogen free (SPF) (Institut für Versuchstierkunde, Berlin, Germany).

Sera which were positive in the NT were subsequently tested by enzyme-linked immunosorbent assay (ELISA). Briefly, microplates coated with p80/120 BVDV antigen (Rhone Merieux GmbH, Laupheim, Germany) were washed and blocked with 1% bovine serum albumin (BSA) for 1 hr at room temperature. Carbonate buffer (0.05 M, pH 9.6) was used as internal negative control instead of p80/120 antigen. The wells were washed and serum dilutions of 1:32 to 1:2,048 were incubated for 1 hr at 37 C. The wells were washed again and incubated with biotin conjugated goat anti-rabbit IgG (Sigma-Chemie GmbH, Deisenhofen, Germany) for 1 hr at 37 C. Following another washing, the conjugate reacted for 1 hr at 37 C with streptavidin peroxidase conjugate (Sigma-Chemie GmbH). After washing, the enzymes were incubated for 15 min with orthophenylene-diamine (OPD) substrate including perhydrol (Sigma-Chemie GmbH). The reaction was stopped with H_2SO_4 and the absorption was measured at 492 nm on a spectrophotometer (Spektra, SLT, Salzburg, Austria). Samples were considered positive when the absorption of the sera, at a dilution ≥ 1:128, was 2.5 fold higher than the internal negative control and two fold higher than the external negative controls (10 SPF rabbits) and when the optical density (OD) exceeded 1.5.

Sera with titers ≥10 against SH9/11 in the NT and two negative sera were studied by H. R. Frey (Institut für Virologie, Hannover, Germany) by a direct neutralizing peroxidase-linked antibody assay (NPLA) according to the method of Hyera (1989).

For the detection of BVDV antigen, spleen samples were homogenized and passed through 0.45 μm filters. Filtrates were inoculated on BVDV-free bovine embryonic lung cells (BEL) and BVDV-free rabbit kidney cells (RK13). The cells were passed three times every 4 days at 37 C, 5% CO_2, 80% humidity and examined regularly for cytopathic effects and by a direct immunofluorescence (IF) assay for non-cytopathic effects (see below). Bovine viral diarrhea virus strain SH9/11 and BVDV strains of bovine origin (NADL Grub 313/83) were used as controls and passed on RK13 cells and BEL cells.

A direct IF assay was used following the procedure of Mayr et al. (1977) applying a fluoresceinisothiocyanate (FITC)-labeled polyclonal anti-BVDV antibody (kindly supplied by P. Steinhagen). Slides with cells containing BVDV (strain SH9/11, NADL and Grub 313/83), and uninfected cells were used as positive and negative controls, respectively.

An antigen ELISA (BioX, Sanofi-Ceva GmbH, Düsseldorf, Germany) was used for detection of BVDV antigen in spleen samples with sufficient material available (n = 19). Samples were regarded as positive when the OD_{450} value of the supernatant, at dilutions ≥1:10 and was >0.3 × OD of the positive control.

Reverse transcription (RT) followed by polymerase chain reaction (PCR) with primers PEST2 (5′-TCAACTCCA-TGTGCCATGTAC-3′) and BVD3 (5′-GTGGACGAGGGCATGCCCA-3′), nt 375–395 and nt 237–255 of the NADL sequence (Collett et al., 1988), respectively,

was used to detect pestivirus specific RNA in 10% of the spleen homogenates, culture supernantants, cultured cells and serum. All experiments were performed in a Gene Amp PCR System 2400 Cycler (Perkin-Elmer, Norwalk, Connecticut). Various methods were used in each step of these experiments; sample preparation: (1) alkaline lysis of tissue (Rolfs et al., 1992), (2) RNeasy total RNA kit (Qiagen, Hilden, Germany), and (3) separation of nucleic acids by proteinase K treatment, organic extraction and ethanol precipitation; RT: (1) Gene Amp EZ rTth RNA PCR kit (Perkin Elmer) using moloney leucemia virus reverse transcriptase (Boehringer, Mannheim, Germany) and primer PEST2 or random hexamer primers, (2) first strand cDNA synthesis kit for RT-PCR with avian myeloblastosis virus -RT (Boehringer) following the manufacturer's instructions, and (3) RT with both enzymes according to the methods of Becher et al. (1996); PCR: with 2 to 5 μl of the RT reaction mixture in 50 μl reactions according: (1) to the Goldstar Polymerase manual (Eurogentec, Seraing, Belgium), (2) to our own protocol, or (3) to protocols recommended by Rolfs et al. (1992) and Becher et al. (1996). As negative controls, RT-PCR experiments were performed without the RT step to check for contaminating DNA in the nucleic acid preparation. Positive controls were performed with BVDV strains NADL and SH9/11.

The frequency proportions of positive sera in the NT-tests were compared by Fisher's exact test (Freeman and Halton, 1951). In these three simultaneous tests, the observed significance level was adjusted according to Bonferroni (Bortz et al., 1990). The Friedman test (Friedman, 1937) was used to evaluate differences in the mean titers among the different virus strains. If significant differences were found in the Friedman-test, a subsequent pairwise comparison of mean titers was performed (Sachs, 1992). The significance level was set to $\alpha = 0.05$. Medians and median absolute deviations were calculated for the antibody titers instead of means and standard deviations. The median absolute deviation (MAD) measures the data dispersion in case of non-normal distributions (Sachs, 1992) and is defined as median absolute deviation (MAD) = median of the set of differences (absolute value of the *i*th titer minus median of all titers).

Of 100 free-ranging rabbits, 40 sera (40%) were positive for NT antibodies to one or more of the three cytopathogenic biotypes of BVDV tested. We detected antibodies against SH9/11 in 40 sera (40%). The NT titers varied between 6 and 13. Antibody titers ≥10 were present in 11% of the sera (28% of positive sera). Antibodies against Grub 313/83 and NADL were found in eight sera (8%). Neutralization test titers against Grub 313/83 varied between 5 and 11. Antibody titers ≥10 were present in two of these sera. Neutralization test titers against NADL varied between 5 and 9. Antibody titers ≥10 were not found. Thirty-two sera which were positive for NT antibodies against SH9/11 were negative for Grub 313/83 and NADL antibodies. The titers and antibody prevalence for SH9/11 were significantly higher compared to Grub 313/92 (titers, Friedman, $P < 0.001$; Sachs, test value > significance threshold; frequencies, Fisher, $P < 0.0001$) and to NADL (titers, Friedman, $P < 0.001$; Sachs, test value > significance threshold; frequencies, Fisher, $P < 0.0001$). In contrast, the titers and prevalences for Grub 313/83 and NADL were not significantly different. For the 40 positive sera, the median of the antibody titers against SH9/11 was 8 (MAD=2) and it was 0 against Grub or NADL (MAD=0).

Of 40 NT positive sera, only 35 were usable for ELISA from which 13 were positive. Twelve of these 13 samples were exclusively positive for SH9/11 in the NT. Eleven sera with titers ≥10 in the NT against SH9/11 and two negative sera were investigated using NPLA. Of these, two sera were positive with titers of 10 and 30, respectively. The remaining sera produced

toxic effects and the tests could not be performed.

No BVDV antigen was detected by antigen ELISA. Moreover, no cytopathic effects were observed in BEL cells or RK13 cells inoculated with spleen homogenates and no virus was isolated. Furthermore, BVDV antigen was not found on RK13 cells after inoculation with homogenized spleens in the direct IF experiments.

Ninty seven spleen samples were tested in RT-PCR experiments for the presence of viral nucleic acids. In animals which had low antibody titers in the NT, different procedures were used to isolate and to amplify viral RNA. Polymerase chain reaction experiments performed with the touch down cycling programme, generally resulted in reduced background amplification (unspecific smear from high to low fragment length). However, none of these experiments resulted in the amplification of the expected approximately 160 bp fragment. Only unspecific fragments with different lengths were obtained from several samples.

Sera from 40 of 100 free-ranging rabbits contained viral neutralizing antibodies against one or more strains of BVDV, indicating that the rabbits either had passively acquired antibodies or had experienced natural infection with BVDV. Moreover, 13 of 35 NT positive sera also were positive for anti-BVDV antibodies as detected by ELISA and at least two positive sera were detected using NPLA. However, virus was not isolated and viral nucleic acids were not detectable in RT-PCR experiments; none of the spleen homogenates were positive by ELISA.

Virus NT titers varied between 5 and 13. These low titers may be due to old infections. It also is possible that rabbits develop a rather weak immune response to pestiviruses. Moreover, group cross-reaction could occur or non-specific inhibitors may have been present (Thrusfield, 1991). However, seroprevalence for BVDV found among the free-ranging rabbits investigated here (40%) is rather high compared to BVDV seroprevalences usually found in deer in Germany (0.5 to 10%) (Weber et al., 1978; Dedek et al., 1988; Frölich, 1995).

Significantly most of the seropositive rabbits and highest titers were detected against BVDV strain SH9/11 rather than against NADL or Grub 313/83. No sera reacted exclusively against NADL or Grub 313/83. Antibodies typically crossreact with different BVDV antigens, but the titers to the homologous antigen are usually higher (Haralambier, 1975; Mayr et al., 1993; Gardner et al., 1996). This suggests that the BVDV-strain to which these rabbits were exposed, is more closely related to SH9/11 in its antigenic structure than to the bovine strains Grub 313/83 or NADL. This is supported by the fact that 12 of 13 sera positive in antibody ELISA reacted only with SH9/11 in the NT. Interestingly, SH9/11 was isolated in a region close to the study area (Frölich and Hofmann, 1995).

This is the first report of a BVDV-like exposure in free-ranging rabbits. However, in transmission experiments, Baker et al. (1954) succeeded in adapting the New York-1 strain of BVDV to domestic rabbits by three alternating calf-to-rabbit passages of splenic material, followed by 75 successive rabbit to rabbit passages. The virus became modified so that inoculation of susceptible calves with material from 75th passage produced only slight signs of disease. This modified virus immunized calves against fully virulent strains. Fernelius et al. (1969) passaged BVDV in adult and neonatal rabbits. Early rabbit-passed virus did not have any effect on rabbits, but in later passages a slightly increased body temperature was noticed at about 2 to 3 days after inoculation (Fernelius et al., 1969). Possibly free-ranging rabbits do not develop clinical signs.

According to the experiments of Baker et al. (1954), the spleen of rabbits contained infective BVDV only up to 5 days post inoculation. In our investigations, virus could not be isolated and no viral nu-

cleic acids could be amplified by RT-PCR. This may be due to the forementioned reason (Baker et al., 1954).

The role of rabbits as carriers of infectious diseases which usually affect Artiodactyla (e.g. malignant catarrhal fever) has been demonstrated (Heuschele et al., 1984; Reid and Buxton, 1984). In conclusion, free-ranging rabbits in northern Germany apparently have been exposed to BVDV. However, reservoir status of these rabbits needs additional exploration.

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