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Isolation of Bovine Viral Diarrhea Virus-like Pestiviruses from Roe Deer (*Capreolus capreolus*)

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ABSTRACT: Cytopathogenic pestiviruses were isolated from two seronegative free-ranging roe deer (*Capreolus capreolus*) from northern Germany (Schleswig-Holstein): an adult female and a young buck collected on 6 December 1990 and 26 July 1991, respectively. The two isolates were identified by polymerase chain reaction as pestiviruses. However, they were negative when primers specific for bovine virus diarrhea virus or classical swine fever virus were used, indicating that the two isolates might belong to a separate group of pestiviruses of wild ruminants different from BVDV.

Key words: *Capreolus capreolus*, roe deer, polymerase chain reaction, virus isolation, electron microscopy, pestivirus, bovine virus diarrhea.

Bovine virus diarrhea virus (BVDV) belongs to the genus *Pestivirus* within the Family Flaviviridae (Horzinek, 1990), and causes a systemic infection affecting a broad range of hosts, including cervids (Holweg, 1987). The primary clinical signs are hemorrhagic mucosal inflammation and general physical impairment (Wiesner, 1987). Signs in cattle include transient acute infections which may be inapparent or mild, or mucosal disease which is inevitably fatal. Transplacental infection will lead to abortion, fetal malformations and development of persistently viremic calves depending on the state of development of the fetus and the biotype (cytopathogenic or noncytopathogenic) of the virus (Brownlie, 1990). Romvary (1965) first isolated a noncytopathogenic BVDV from the spleen of a roe deer (*Capreolus capreolus*) living in an area where BVDV-infected cattle were kept. In transmission experiments, this virus strain caused diarrhea in a calf (Romvary, 1965). Schellner (1977) isolated BVDV from spleen, intestinal lymph nodes, and abomasal mucosa of roe deer suffering from abomasitis and severe enteritis. Neumann et al. (1980), Weber et al. (1982), and Diaz et al. (1988) isolated the virus from farmed fallow deer (*Dama dama*). Isolation of a noncytopathogenic BVDV from the spleen of a red deer (*Cervus elaphus*) was reported by Nettelton et al. (1980). A pestivirus differing from BVDV was isolated from red deer by Baradel et al. (1988). Our objective was to characterize two cytopathogenic pestiviruses isolated from seronegative roe deer.

Spleen samples from 203 cervids (113 roe deer, 30 red deer, 61 fallow deer) shot by hunters, collected between November 1990 and October 1992 were examined for BVDV in the course of a serological survey. Animals originated from various hunting areas and nine wildlife parks in Schleswig-Holstein (54°30′ to 54°50′N, 9°20′ to 9°40′E), Berlin (52°30′N, 13.20′E) and Bavaria (48°00′ to 48°50′N, 9°30′ to 13°10′E), Germany. Spleen samples were stored at −70 C, homogenized, and passed through 0.45 μm filters. Filtrates were inoculated on BVDV-free bovine embryonic lung cells (BEL). The cells were passed in Dulbecco's Modified Eagle Medium with 10% fetal calf serum (Life Technologie GmbH, Berlin, Germany) three times every 4 days at 37 C, 5% CO_2, 80% humidity and examined regularly for cytopathic effects.

A direct immunofluorescence assay was used following the procedure of Mayr et al. (1977). Briefly, the BEL cells from the third passage after sample inoculation were grown on coverslips, fixed in acetone, and subsequently incubated with a polyclonal anti-BVDV antibody labeled with fluoresceinisothiocyanate (FITC) (Serva Feinbiochemica GmbH, Heidelberg, Germany). The antibody was produced in a rabbit

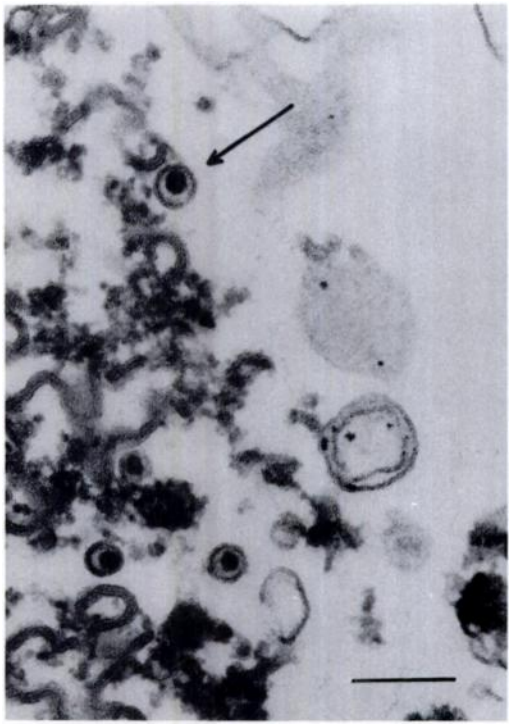

FIGURE 1. Electron microscopic images of the two pestivirus isolates from roe deer. Virus particles (arrow) in BVDV-free bovine embryonic lung cells are characteristic of pestiviruses. Bar = 200 nm.

exposed to BVD strains isolated from a cow in Schleswig-Holstein (54°04′N, 9°58′E). Slides with cells containing BVDV (strain NADL), and uninfected cells were used as positive and negative controls, respectively (Mayr et al., 1977).

The reverse transcriptase polymerase chain reaction (RT-PCR) as described by Wirz et al. (1993) was used for the detection of pestivirus-specific RNA in organs, except that primer BVD3 (Microsynth, Windisch, Switzerland) was used instead of Pest2 (Microsynth) as the second primer, allowing the amplification of a DNA fragment of approximately 155 base pairs instead of 75 base pairs as with primer Pest2. Briefly 10% (w/v) suspensions of spleen samples in phosphate buffered saline were homogenized and clarified. First strand complementary DNA (cDNA) synthesis was done with reverse transcriptase and an antisense primer (Pest2) following heat denaturation of the sample. Polymerase chain reaction amplification of the cDNA was carried out with Taq DNA polymerase (P. H. Stehelin and Cie A. G., Basel, Switzerland) for 35 cycles after the addition of a second (sense-oriented) primer (BVD3). The amplified DNA was analyzed by electrophoresis in a 2% agarose gel. Positive samples were characterized further using BVDV-specific (Lopez 1/2, Microsynth) (Lopez et al., 1991) and classical swine fever virus (CSFV)-specific primers (HCV 1/2, Microsynth) (Wirz et al., 1993).

Electron microscopy was performed in collaboration with Dr. Gelderblom at the Robert Koch Institute, Berlin. Infected cells were fixed in 2.5% glutaraldehyde (Sigma-Chemie, Deisenhoven, Germany) and ultra-thin sections were prepared as described by Gelderblom et al. (1987).

Cytopathogenic pestiviruses were isolated from two of the 203 samples of free-ranging roe deer from northern Germany (Schleswig-Holstein): an adult female (54°35′N, 9°22′E) and a young buck (54°45′N, 9°30′E) collected on 6 December 1990 and 26 July 1991, respectively. Both sera had no specific antibodies against BVDV (Frölich, 1994). All other spleen samples were negative. Both viral isolates were positive in the direct immunofluorescence test. Virus particles characteristic for pestiviruses were found in tissue cultures examined by electron microscopy (Fig. 1). Anti-BVDV antiserum from cattle neutralized the infectivity of the isolates and the cytopathic effect of the isolates resembled that of pestiviruses (Holweg, 1987). The two isolates were identified as pestiviruses in the RT-PCR using the two pan-pestivirus-specific primers Pest2 and BVD3 (Fig. 2). However, they were negative when primers specific for BVDV (Lopez 1/2) or CSFV (HCV 1/2) were used.

These are the first known cytopathogenic pestiviruses from roe deer. Romvary (1965) isolated a noncytopathogenic BVDV from the spleen of a roe deer in Hungary.

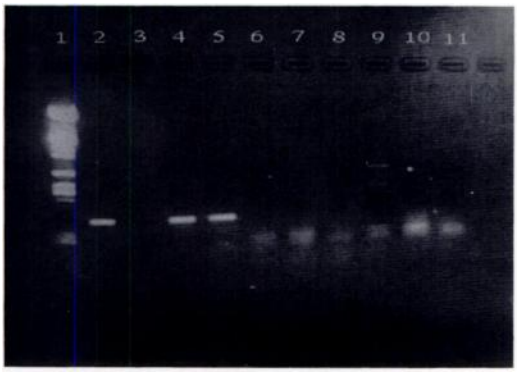

FIGURE 2. Agarose gel electrophoresis. Reverse transcriptase polymerase chain reaction with pestivirus-specific primers (PEST 2/BVD 3). 1: DNA-marker (p Bluescript-Sau 3A); 2: positive control (CSFV Alfort); 3: negative control; 4: sample SH9 (pestivirus-positive); 5: sample SH11 (pestivirus-positive); 6 to 11: pestivirus-negative samples.

A cytopathogenic BVDV in wild ruminants was isolated from a giraffe (*Giraffa camelopardalis*) in Kenya by Plowright in 1969 (Hamblin and Hedger, 1979).

If the pathogenesis in deer is similar to cattle, the two isolates from roe deer probably were taken from individuals who were in the early stage of mucosal disease or had an acute transient form of BVD. Cattle are seronegative at this stage; acute BVD is followed later by a seroconversion leading to immunity (Brownlie, 1990). In the case of mucosal disease, animals are persistently infected with a noncytopathogenic BVDV against which they are immunotolerant, and concurrently are infected with a cytopathogenic strain most likely originating from a mutation of the original infecting noncytopathogenic virus (Brownlie, 1990).

In addition to classical virologic methods, the two isolates were identified by means of PCR as pestiviruses. However, they were negative when BVDV- or CSCF-specific primers were used. This is evidence that the genome of the two isolates differs from BVDV and CSVF at least in the regions amplified by PCR. The two isolates therefore cannot be classified within the BVDV or CSVF subgroup of pestiviruses and they probably are members of a separate group of pestiviruses of wild ruminants. Three prototype viruses represent the genus pestivirus, namely BVDV, CSVF, and border disease virus (BDV) of sheep, each with different strains. The assignment of the two isolates to a particular pestivirus strain is not possible at present. Further analysis of the genome of the two isolates is needed to determine whether they are members of a separate group of pestiviruses of wild ruminants or whether they are similar to known strains.

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