ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

VIROLOGY

Virology 311 (2003) 96–104

www.elsevier.com/locate/yviro

# Genetic and antigenic characterization of novel pestivirus genotypes: implications for classification

Paul Becher,[a,*] Ramiro Avalos Ramirez,[a] Michaela Orlich,[a] Sibilina Cedillo Rosales,[a] Matthias König,[a] Matthias Schweizer,[b] Hanspeter Stalder,[b] Horst Schirrmeier,[c] and Heinz-Jürgen Thiel[a]

[a] *Institut für Virologie (FB Veterinärmedizin), Justus-Liebig-Universität, Frankfurter Straβe 107, D-35392 Giessen, Germany*
[b] *Institut für Veterinär-Virologie, Universität Bern, CH-3012 Bern, Switzerland*
[c] *Institut für Diagnostische Virologie, BFAV, D-17498 Insel Riems, Germany*

Received 1 November 2002; returned to author for revision 16 December 2002; accepted 2 January 2003

**Abstract**

Currently, the genus *Pestivirus* comprises the four approved species *Bovine viral diarrhea virus 1* (BVDV-1), BVDV-2, *Classical swine fever virus* (CSFV), and *Border disease virus (BDV)* and one tentative fifth species represented by a single strain (H138) isolated from a giraffe in Kenya more than 30 years ago. To further address the issue of heterogeneity of pestiviruses we have determined the entire $N^{pro}$ and E2 coding sequences for several new pestivirus isolates. Interestingly, phylogenetic analysis revealed that one pestivirus isolated in the 1990s in Africa is closely related to strain H138. Moreover, several novel pestiviruses isolated from sheep group together with the previously described strain V60 (Reindeer-1) isolated from a reindeer, whereas one ovine pestivirus strain (Gifhorn) significantly differs from all previously described pestiviruses, including BDV. We propose to term these mainly sheep-derived pestiviruses BDV-2 (V60-like isolates) and BDV-3 (Gifhorn); consequently, the "classical" BDV isolates should be termed BDV-1. As an additional criterion for segregation of pestiviruses, the antigenic relatedness of pestivirus isolates covering all observed major genotypes was studied by cross-neutralization assays. Analysis of the antigenic similarities indicated the presence of seven major antigenic groups corresponding to BVDV-1, BVDV-2, CSFV, BDV-1, BDV-2, BDV-3, and "giraffe". Taking into account the host origin, the lack of differences concerning the course of disease, and the results of our genetic and antigenic analyses, we suggest that BDV-1, BDV-2, and BDV-3 should be considered as major genotypes within the species BDV.
© 2003 Elsevier Science (USA). All rights reserved.

## Introduction

The genus *Pestivirus* in the family *Flaviviridae* currently contains the four accepted species *Bovine viral diarrhea virus-1* (BVDV-1), BVDV-2, *Classical swine fever virus* (CSFV), and *Border disease virus* (BDV) and one tentative species represented by a single pestivirus isolated from a giraffe (Becher and Thiel, 2002; Heinz et al., 2000). Moreover, a pestivirus obtained from reindeer (Reindeer-1) may represent either an additional species within the genus or a distantly related subgroup of BDV (Avalos-Ramirez et al., 2001; Becher et al., 1999). Traditionally, pestiviruses are named after the affected host species and the diseases they cause. BVDV-1, BVDV-2, and BDV are, however, able to cross species barriers to infect a wide range of hosts within the *Artiodactyla* (Becher et al., 1999; Becher et al., 1997; Doyle and Heuschele, 1983; Hamblin and Hedger, 1979; Nettleton, 1990; Plowright, 1969). In contrast, the natural host range of CSFV is restricted to domestic pigs and wild boars. Pestiviruses are important pathogens of cattle, sheep, and pigs and cause significant economic losses worldwide. Pestivirus infections can have different consequences, such as fertility problems, immunosuppression, diarrhea, thrombocytopenia, and, frequently, inapparent courses (Baker, 1987; Thiel, Plagemann, and Moennig, 1996). In pregnant animals, transplacental infection can lead to abortion, still-

* Corresponding author. Fax: +49-641-99-38359.
*E-mail address:* paul.becher@vetmed.uni-giessen.de (P. Becher).

0042-6822/03/$ – see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0042-6822(03)00192-2

Table 1
Pestivirus isolates

| Virus isolate | Abbreviation | Year of isolation | Species of origin | Region of isolation | GenBank accession No. | |
|---|---|---|---|---|---|---|
| | | | | | $N^{pro}$ | E2 |
| PG-2 | — | 1990s[a] | Cattle? | Africa | AY163647 | AY163654 |
| V2536/2 | V2536 | 1994 | Sheep | United Kingdom | AY163648 | AY163655 |
| T1802/1 | T1802 | 1992 | Sheep | United Kingdom | AY163649 | AY163656 |
| 466/T/85 | 466 | 1985 | Sheep | Germany | AY163650 | AY163657 |
| 17385/00 | 17385 | 2000 | Sheep | Germany | AY163651 | AY163658 |
| Chemnitz AZ 79 | AZ79 | 1999 | Sheep | Germany | AY163651 | AY163659 |
| Gifhorn | — | 1999 | Sheep | Germany | AY163653 | AY163660 |

[a] Year of isolation unknown.

birth, malformation, and persistent infection of the offspring.

Pestiviruses have positive-stranded, nonpolyadenylated RNA genomes, usually with a length of approximately 12.3 kb (Avalos-Ramirez et al., 2001; Becher, Orlich, and Thiel, 1998; Collett et al., 1988; Meyers, Rümenapf, and Thiel, 1989; Ridpath and Bolin, 1995). The genomic RNA contains one large open reading frame flanked by 5′ and 3′ nontranslated regions (NTR). In the virus-encoded polyprotein, the viral proteins are arranged in the following order (from the N to the C terminus): $N^{pro}$, C, $E^{ms}$, E1, E2, p7, NS2-3, (NS2, NS3), NS4A, NS4B, NS5A, NS5B (reviewed in Thiel, Plagemann, and Moennig, 1996); the abbreviation $N^{pro}$ refers to an N-terminal autoprotease and $E^{ms}$ (ribonuclease secreted) to a glycoprotein with ribonuclease activity. The structural proteins are represented by the capsid protein C and three envelope proteins ($E^{ms}$, E1, and E2). The remaining proteins are presumably nonstructural (NS).

The genetic relatedness of pestivirus isolates, including their relationship to the type viruses of the species, is one important parameter for classification of pestiviruses (Heinz et al., 2000). In addition, pestivirus species demarcation considers the reactivity with defined serological reagents, including binding assays with monoclonal antibodies and cross-neutralization tests with polyclonal antisera (Avalos-Ramirez et al., 2001; Cay et al., 1989; Dekker, Wensvoort, and Terpstra, 1995; Paton et al., 1995). Furthermore, differences in natural host range, disease, and pathology may assist in the classification of pestiviruses (Heinz et al., 2000). This report deals with the genetic and antigenic characterization of several new pestiviruses. The results of our comparative analyses revealed the detection of two novel genotypes comprising mainly sheep-derived pestiviruses.

## Results and discussion

### *Analysis of $N^{pro}$ and E2 coding sequences*

Genetic comparison of pestiviruses has been the subject of several studies (Becher et al., 1994, 1995; Couvreur et al., 2002; Nagai et al., 2001; Paton et al., 1995; Pellerin et al., 1994; Ridpath, Bolin, and Dubovi, 1994; Vilcek et al., 1997, 2001). Most frequently partial sequences of the 5′ NTR were analyzed resulting in the availability of more than 1000 pestivirus 5′ NTR sequences. However, statistical analyses of phylogenetic trees based on partial 5′ NTR sequences revealed that clustering within the species and in some cases even assigning of pestiviruses into species were ambiguous (Becher et al., 1997). It has been concluded that partial sequences from the 5′ NTR can be used for the segregation of pestivirus isolates into the established species but are less suited to a further subdivision of the virus species into defined subgroups (Becher et al., 1997; Heinz et al., 2000). Alternatively, segregation of pestiviruses into species and subgroups on the basis of complete $N^{pro}$ and E2 coding genes is supported by significantly high confidence levels (Becher et al., 1997, 1999). Accordingly, these regions are well suited for phylogenetic analyses of pestiviruses. Both the $N^{pro}$ and E2 genes are addressed here.

To study the genetic relationship of novel pestivirus isolates we first concentrated on the $N^{pro}$ coding region of the genome. For all pestiviruses analyzed so far, including the ones listed in Table 1, reverse transcription–polymerase chain reaction (RT–PCR) using a single primer pair allowed the amplification of the $N^{pro}$ gene together with flanking sequences. After molecular cloning in a bacterial vector the entire $N^{pro}$ coding sequences were determined by sequencing three independent clones. The obtained sequences were first subjected to pairwise comparisons and aligned with corresponding published sequences of pestivirus reference strains (data not shown). For all pestiviruses, including the novel virus isolates presented here, the $N^{pro}$ coding gene comprises 504 nucleotides. The evolutionary relationships of the $N^{pro}$ genes were estimated by phylogenetic analysis using the neighbor-joining method (Felsenstein, 1993). In addition to the sequences obtained for the ovine pestivirus isolates V2536, T1802, 466, 17385, AZ79, and Gifhorn, and isolate PG-2 (Table 1), several published sequences of pestivirus reference strains were included. Bootstrap resampling of phylogenetic trees was carried out to test the robustness of the observed clades. Bootstrap values obtained for the single pestivirus species as well as for subgroups

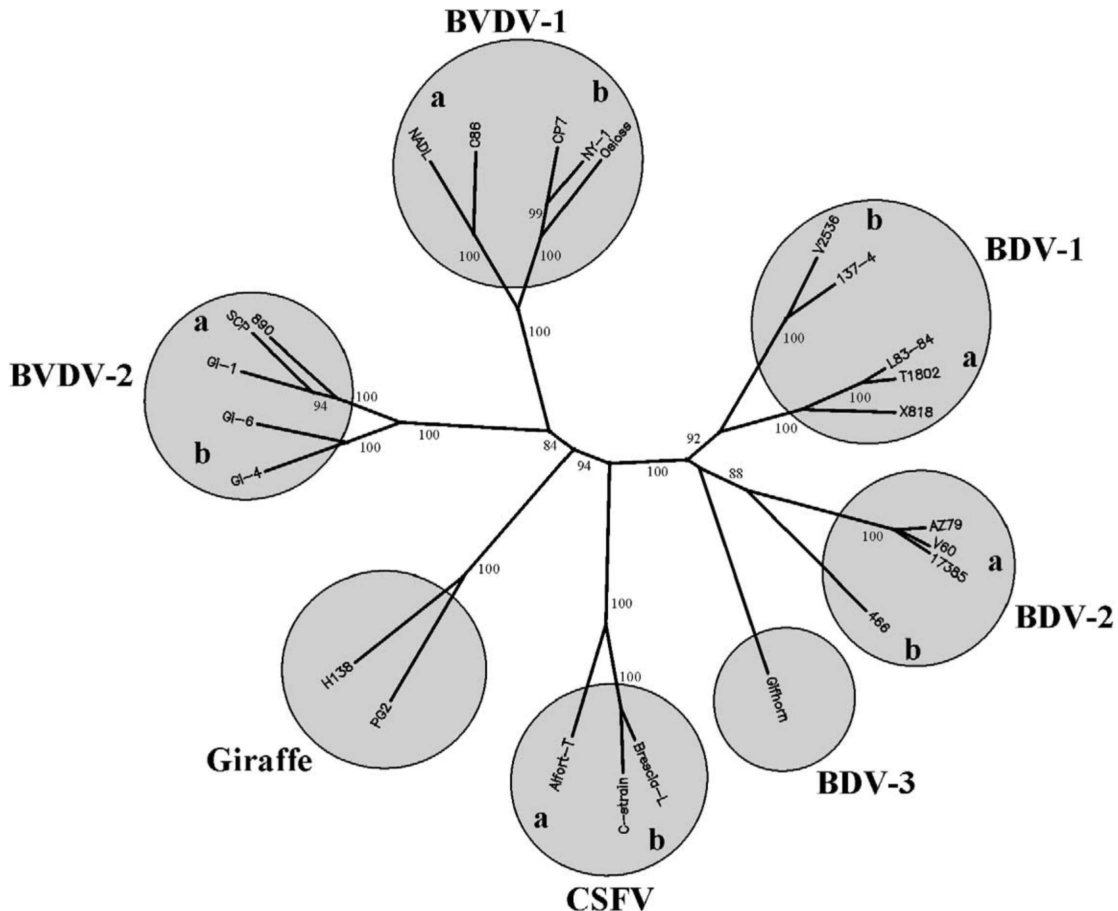


Fig. 1. Dendrogram showing the genetic relationship of pestivirus strains. The phylogenetic tree was constructed from the complete N$^{pro}$ coding sequences. Sequences of isolate PG-2 and ovine strains V2536, T1802, 466, 17385, AZ79, and Gifhorn were generated in this study. Other sequences were obtained from the GenBank database. Multiple sequence alignments were computed by PILEUP included in the GCG software package. Distances were calculated by the Kimura 2-parameter method (Kimura, 1980) and used to construct the tree according to the neighbor-joining method (Felsenstein, 1993). Branch lengths are proportional to genetic distances. Numbers indicate the percentage of 1000 bootstrap replicates that support each labeled interior branch (Felsenstein, 1985; Hedges, 1992). This is an unrooted tree.

within the species were >88% and in most cases 100%. The phylogenetic tree shows seven major branches corresponding to (i–iv) the pestivirus species BVDV-1; BVDV-2; BDV; CSFV; (v) the tentative species represented by strain H138 from giraffe; (vi) one additional group comprising the ovine pestivirus isolates 466, 17385, AZ79, and the previously reported pestivirus strain V60 isolated from reindeer (Becher et al., 1999); and (vii) the ovine pestivirus isolate Gifhorn (Fig. 1). BVDV-1 and BVDV-2 have bifurcated from a common branch and BDV and the two novel groups of mainly sheep-derived pestiviruses from another.

Interestingly, pestivirus strain PG-2 isolated in Africa in the 1990s is closely related to the previously reported strain H138 obtained from a giraffe in Kenya more than 30 years ago. This is the first report on identification of an H138-like pestivirus. The detection and characterization of PG-2 suggests that similar pestiviruses are present in Africa. Although only little is known about natural host range, geographic distribution, and virulence of H138-like viruses, the transmission of such novel pestiviruses to cattle and sheep may represent a serious danger for the lifestock industry. Accordingly, characterization of such exotic pestiviruses is important for diagnosis and control of diseases caused by pestiviruses.

Surprisingly, the N$^{pro}$ sequences of the ovine isolates Gifhorn, 466, 17385, and AZ79 are clearly different from corresponding sequences of the established pestivirus species, including all BDV strains reported so far; the latter three isolates are closely related to pestivirus strain V60 obtained from a reindeer (Fig. 1). Accordingly, our analysis of pestiviruses isolated from sheep led to the identification of two novel major genetic groups in addition to previously reported ovine virus isolates belonging to the species BVDV-1, BVDV-2, and BDV (Becher et al., 1994, 1995; Roehe, Woodward, and Edwards, 1992; Sullivan et al., 1994; Vilcek et al., 1997). Taking into account the host range, the lack of differences concerning disease, and the results of the phylogenetic analysis, we suggest to term these two additional groups of mainly sheep-derived pestiviruses BDV-2 (isolates 466, 17385, AZ79, and V60) and

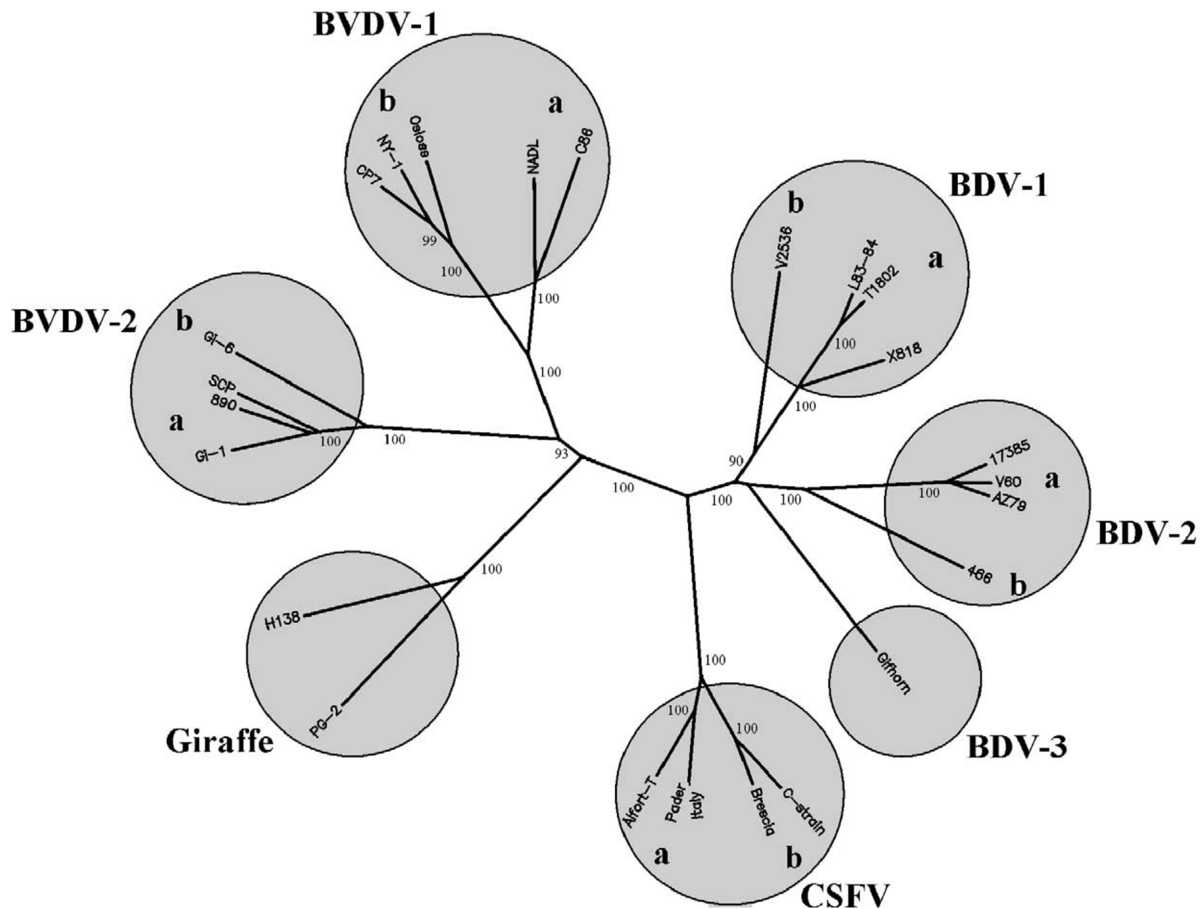


Fig. 2. Dendrogram showing the genetic relationship of pestivirus strains. The phylogenetic tree was constructed from the complete E2 coding sequences using the same methods as described in the legend to Fig. 1. Sequences of isolate PG-2 and ovine strains V2536, T1802, 466, 17385, AZ79, and Gifhorn were generated in this study. Other sequences were obtained from the GenBank database. Branch lengths are proportional to genetic distances. Numbers indicate the percentage of 1000 bootstrap replicates that support each labeled interior branch (Felsenstein, 1985; Hedges, 1992). This is an unrooted tree.

BDV-3 (isolate Gifhorn). Consequently, the "classical" BDV isolates, including reference strain X818 and isolates V2536 and T1802, should be termed BDV-1 (Fig. 1).

BDV-1, BDV-2, BVDV-1, BVDV-2, and CSFV can be divided into defined genetic subgroups tentatively termed BDV-1a and -b, BDV-2a and -b, BVDV-1a and -b, BVDV-2a and -b, and CSFVa and -b, respectively (Fig. 1). It should be noted that additional subgroups of BVDV-1 have been reported (Becher et al., 1997, 1999; Vilcek et al., 2001). Phylogenetic analyses of a larger collection of pestiviruses, including members of these additional subgroups, revealed no differences concerning the segregation of the pestivirus isolates analyzed here (data not shown).

The phylogenetic analysis indicated that pestiviruses can be divided into defined species and subgroups by the branching order of the phylogenetic tree (Fig. 1). As an additional method, the sequences can be compared by pairwise sequence similarity. To define ranges of sequence similarity for the $N^{pro}$ coding sequences, the respective pairwise evolutionary distances were calculated. Considering BDV-1, BDV-2, and BDV-3 as separate species, the ranges of sequence divergence were 4.1–13.8% between virus isolates of one subgroup, 16.4–27.7% between subgroups, and 28.9–53.8% between species (data not shown). It should be noted that the distances between BDV-1, BDV-2, and BDV-3 were 28.9–37.6% and thus intermediate between the ranges calculated between subgroups and between the established species, including "giraffe" (36.2–53.8%).

In addition to the $N^{pro}$ region, the genomic region encoding the complete protein E2 was analyzed. The structural glycoprotein E2 represents one of the most variable proteins of pestiviruses and is able to induce virus neutralizing antibodies and protection. The E2 gene sequences of BDV-1 strains V2536 and T1802; BDV-2 strains 466, 17385, and AZ79; BDV-3 strain Gifhorn; and pestivirus strain PG-2 were determined in this study and compared with the sequences of other pestivirus strains previously reported. To study the heterogeneity within the E2 region, a phylogenetic tree was generated using the same methods as described for the $N^{pro}$ region (Fig. 2). This tree demonstrates the segregation of pestiviruses into seven major genetic groups: BVDV-1, BVDV-2, CSFV, BDV-1, BDV-2, BDV-3, and "giraffe" (Fig. 2). Accordingly, phylogenetic analysis of the

Table 2
Cross-neutralization titers[a] of antisera raised against different pestivirus strains

| Antiserum against strain | | Pestivirus strain | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | NY-1 | C86 | 890 | Gi-2 | Italy | Pader | X818 | L83/84 | V2536 | 17385 | V60 | Gifhorn | H138 |
| **BVDV-1** | **NY-1** | **4096** | 768 | 80 | 64 | 512 | 256 | 128 | 96 | 64 | 52 | 48 | 240 | 128 |
| | **C86** | 40 | **128** | 10 | 8 | 8 | — | — | 8 | — | — | — | — | — |
| **BVDV-2** | **890** | 48 | 28 | **640** | 512 | 8 | — | — | 8 | 8 | 8 | — | — | — |
| | **Gi-2** | — | — | 192 | **192** | — | — | — | — | — | — | — | — | — |
| **CSFV** | **Italy** | — | — | — | — | **768** | 768 | 20 | 12 | 48 | 8 | 16 | 8 | — |
| | **Pader** | — | — | — | — | 896 | **896** | 40 | 38 | 24 | 32 | 14 | 16 | — |
| **BDV-1** | **X818** | 28 | 20 | — | — | 256 | 320 | **2560** | 512 | 352 | 352 | 192 | 224 | — |
| | **L83/84** | 14 | — | — | — | 96 | 112 | 1024 | **1280** | 120 | 224 | 64 | 58 | — |
| | **V2536** | 16 | 12 | — | — | 208 | 384 | 160 | 208 | **640** | 136 | 46 | 176 | 22 |
| **BDV-2** | **17385** | — | — | — | — | 20 | 60 | 128 | 112 | 48 | **320** | 96 | 36 | — |
| | **V60** | — | — | — | — | 80 | 96 | 96 | 160 | 28 | 480 | **1280** | 40 | — |
| **BDV-3** | **Gifhorn** | 22 | 32 | — | — | 40 | 216 | 208 | 224 | 192 | 236 | 88 | **1962** | 10 |
| **Giraffe-1** | **H138** | — | — | — | — | — | — | — | — | — | — | — | | **224** |

[a] Reciprocal of the highest dilution of serum capable of completely neutralizing 100–300 $TCID_{50}$ of the respective virus strain; homologous titers in bold; -, titer <8.

E2 region confirmed the subdivision of the genus *Pestivirus* as determined by analysis of the $N^{pro}$ region (Figs. 1 and 2). Moreover, the branching order of phylogenetic trees obtained by analyses of the E2 and $N^{pro}$ protein sequences was identical to the ones obtained with the nucleotide sequences but supported by slightly lower bootstrap values (data not shown). Similar to the results obtained with the $N^{pro}$ coding sequences, the genetic distances between BDV-1, BDV-2, and BDV-3 were in most cases intermediate between the ranges calculated between established species and between subgroups (data not shown).

Taken together, the topology of the phylogenetic trees and the analyses of the ranges of sequence similarity suggest the presence of seven major genetic groups, namely BVDV-1, BVDV-2, BDV-1, BDV-2, BDV-3, CSFV, and "giraffe" (Figs. 1 and 2). Although BDV-1, BDV-2, and BDV-3 are more closely related to each other than to other pestiviruses, the genetic distances between these three major genetic groups are significantly higher than the ones observed between subgroups of established pestivirus species. Accordingly, BDV-1, BDV-2, and BDV-3 can be classified either as distinct species or as major genotypes of BDV. To clarify the taxonomic status of BDV-1, BDV-2, and BDV-3, the antigenic relatedness of pestiviruses was studied.

### *Antigenic relatedness of pestiviruses by cross-neutralization*

In addition to nucleotide sequence relatedness, the reactivity of pestivirus isolates with defined serological reagents represents a further important criterion for species demarcation (Heinz et al., 2000). Serological assays, in particular cross-neutralization tests, have been widely used to demonstrate similarities as well as differences between pestiviruses (Dekker, Wensvoort, and Terpstra, 1995; Howard, Brownlie, and Clarke, 1987; Paton et al., 1995; Pellerin et al., 1994). Recently, we compared 10 pestivirus isolates, including BVDV-1, BVDV-2, BDV, CSFV, H138 (Giraffe-1), and V60 (Reindeer-1) in cross-neutralization tests (Avalos-Ramirez et al., 2001). This analysis resulted in a clear separation of six major antigenic groups reflecting the genetically defined segregation of pestiviruses. In the present study three additional antisera were generated after infection of sheep with BDV-1 strain V2536, BDV-2 strain 17385, and BDV-3 strain Gifhorn and used in cross-neutralization studies.

The neutralizing capacity of each of the 13 antisera was determined against each pestivirus isolate (Table 2). The neutralizing antibody titers against members of heterologous pestivirus groups were significantly lower than against members of the homologous group. BVDV-1 and BVDV-2 were poorly or not neutralized by antisera raised against CSFV, BDV-1, BDV-2, BDV-3, and H138 (Table 2). Interestingly, the antisera raised against BDV-1 strains X818, L83-84, and V2536; BDV-2 strains 17385 and V60; and BDV-3 strain Gifhorn neutralized heterologous BDV strains and CSFV to the same extent. The giraffe pestivirus was not or only poorly neutralized by the heterologous antisera employed. With the exception of pestivirus strain PG-2 (data not shown), all other pestiviruses included in this study were not neutralized by the antiserum raised against H138 (Table 2).

For further characterization of the antigenic relatedness, the coefficient of antigenic similarity ($R$) was calculated for each pair of viruses (Table 3) (Archetti and Horsfall, 1950).

Table 3
Coefficients of antigenic similarity (*R*)

| Pestivirus strain | | C86 | 890 | Gi-2 | Italy | Pader | X818 | L83/84 | V2536 | 17385 | V60 | Gifhorn | H138 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BVDV-1 | NY-1 | 24.2 | 3.8 | 0.9 | 1.3 | 0.8 | 1.8 | 1.6 | 1.9 | 0.6 | 0.3 | 2.5 | 1.2 |
| | C86 | | 5.8 | 1.8 | 0.9 | 0.3 | 0.8 | 0.7 | 1.2 | 0.5 | 0.2 | 1.1 | 0.6 |
| BVDV-2 | 890 | | | 89.4 | 0.4 | 0.1 | 0.1 | 0.3 | 0.4 | 0.6 | 0.1 | 0.1 | 0.3 |
| | Gi-2 | | | | 0.3 | 0.2 | 0.1 | 0.2 | 0.3 | 0.4 | 0.2 | 0.1 | 0.5 |
| CSFV | Italy | | | | | 100.0 | 5.1 | 3.4 | 14.3 | 2.6 | 3.6 | 1.5 | 0.2 |
| | Pader | | | | | | 7.5 | 6.1 | 12.7 | 8.2 | 3.4 | 4.4 | 0.2 |
| BDV-1 | X818 | | | | | | | 40.0 | 20.1 | 25.0 | 7.5 | 10.4 | 0.1 |
| | L83/84 | | | | | | | | 17.4 | 24.7 | 7.9 | 7.2 | 0.2 |
| | V2536 | | | | | | | | | 17.8 | 4.0 | 16.4 | 1.2 |
| BDV-2 | 17385 | | | | | | | | | | 33.9 | 12.2 | 0.4 |
| | V60 | | | | | | | | | | | 3.8 | 0.2 |
| BDV-3 | Gifhorn | | | | | | | | | | | | 0.5 |

Significant antigenic differences are indicated by *R* values of ≤25 representing ≥4-fold differences in titers of homologous and heterologous antisera. The *R* values were used to construct a dendrogram that shows the antigenic relatedness of pestiviruses (Fig. 3). To estimate the similarity between groups of strains, the respective correlation coefficients were averaged. The dendrogram shows that pestiviruses segregate into seven major antigenic groups corresponding to the genetically defined major groups (Fig. 3). The averaged *R* values between these major antigenic groups are <14.5 (representing >6.9-fold differences in titers of homologous and heterologous antisera). In agreement with the results obtained from the genetic analysis, BDV-1, BDV-2, and BDV-3 are antigenically more similar to each other (average *R* values range from 10 to 14.5) than to other pestivirus species (average *R* values <8.5).

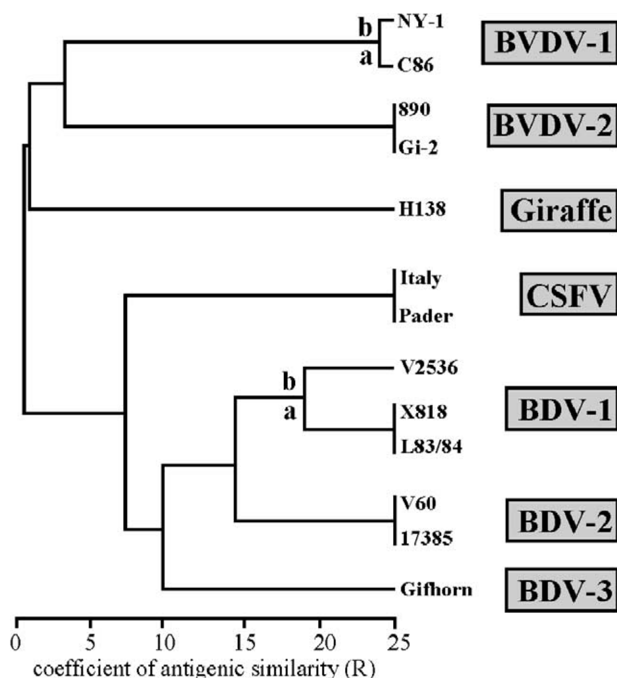


Fig. 3. Antigenic diversity of pestiviruses. The dendrogram shows the serological relationships between 13 pestivirus isolates covering all observed major genetic groups by using the coefficients of antigenic similarity (*R* values) between pairs of pestivirus isolates (Table 3). To evaluate the relatedness between groups of virus isolates the *R* values were averaged. *R* values of <25 represent >fourfold differences in titers with homologous and heterologous antisera and indicate significant antigenic differences (Hubalek, 1982). *R* values of >25 are considered not significant and are therefore not drawn to scale.

Furthermore, significant antigenic differences were found between subgroups of BVDV-1 (BVDV-1a and -b) and BDV-1 (BDV-1a and -b); the respective *R* values were 17.4 and 24.2 (representing 4.1-fold and 5.8-fold differences), respectively (Table 3). Recently, significant antigenic differences between BVDV-1 isolates from different subgroups (as determined by cross-neutralization assays) have also been reported by others (Couvreur et al., 2002; Nagai et al., 2001). The respective *R* values were not provided in these studies and were therefore calculated by us to allow comparison with our data. Remarkably, for several pairs of BVDV-1 isolates, the *R* values were <10 (representing >10-fold differences); the lowest *R* value calculated for a pair of BVDV-1 isolates was 1.9 (representing >50-fold difference). In general, several studies demonstrated that BVDV-1 isolates are antigenically more closely related to each other than to BVDV-2 (Avalos-Ramirez et al., 2001; Couvreur et al., 2002; Nagai et al., 2001; Pellerin et al., 1994). However, in some cases the antigenic differences between BVDV-1 strains of different subgroups can reach values similar to those found between BVDV-1 and BVDV-2. The detection of such pronounced antigenic differences between individual BVDV-1 isolates is important for diagnosis and control of diseases caused by BVDV and should be taken into account with regard to development of efficacious BVDV vaccines.

### *Implications for classification*

In summary, our genetic and antigenic analyses demonstrate that BDV-2 and BDV-3 represent additional novel groups of pestiviruses different from the established species and that isolate PG-2 is closely related to the giraffe strain

H138. The latter currently represents the only member of a tentative pestivirus species. Analyses of PG-2 and H138 show that they belong to one pestivirus species that should be considered as an approved member of the genus *Pestivirus* (Figs. 1–3). Although putative names for this additional species have to be discussed among pestivirologists, we suggest the term *Giraffe Pestivirus.* Furthermore, BDV-1, BDV-2, and BDV-3 can be classified either as distinct species or as major genotypes of one species. According to (i) the fact that BDV-1, BDV-2, and BDV-3 are genetically and antigenically more closely related to each other than to other pestivirus species; (ii) lack of apparent differences concerning the course of disease; and (iii) host range, we propose that BDV-1, BDV-2, and BDV-3 should be considered three genotypes of one species (BDV). These criteria outlined above for BDV also apply to BVD viruses, which are currently divided into two species. In light of our proposal for BDV, we suggest that BVDV-1 and BVDV-2 should be two genotypes within one species.

## Materials and methods

### *Virus isolates and cells*

The pestivirus isolate PG-2 was isolated from a bovine cell culture infected with *Theileria sp.* in the 1990s in Africa; it is not known whether the bovine cells were derived from an infected cattle or were contaminated during propagation. The ovine isolates 466, 17385, Chemnitz AZ79, and Gifhorn were obtained from sheep with border disease in Germany. Isolates 466 and Chemnitz AZ79 have been described previously (Schaarschmidt et al., 2000; Waldvogel et al., 1995). Isolates Gifhorn and 17385 were obtained in 1999 and 2000, respectively. BVDV-1 strains C86 and NY-1; BVDV-2 strains 890 and Gi-2; BDV strains X818, L83/84, T1802, and V2536; CSFV strains Italy and Pader as well as the pestivirus strains V60 (Reindeer-1) isolated from reindeer (*Rangifer tarandus*) and H138 (Giraffe-1) isolated from giraffe (*Giraffa camelopardalis*) have been described (Avalos-Ramirez et al., 2001; Becher et al., 1994, 1997; Plowright, 1969; Ridpath and Bolin, 1995; Vilcek et al., 1997). Ruminant pestiviruses were multiplied on Madin–Darby bovine kidney (MDBK) cells obtained from the American Type Culture Collection (Rockville, MD, USA), whereas porcine PK-15 cells were used for infection with CSFV isolates. Propagation and infection of cells was carried out as described (Becher et al., 1997).

### *Oligonucleotides*

Oligonucleotides were purchased from MWG Biotech GmbH (Ebersberg, Germany). The sense primers OL 100 (located in the 5′ NTR) and OL P2250 (located in the E1 coding region) as well as the antisense primers OL 1400R (located in the $E^{ms}$ coding region) and OL RP7R1 (located in the p7 coding region) have been described previously (Becher et al., 1996, 1999; Becher, Orlich, and Thiel, 1998). Sequences of other oligonucleotides and their polarities are as follows: OL BDV2240 (5′-GTGGCCMTATGAAA-CAACC-3′, corresponding to nucleotides 2241–2259 of the genomic sequence of BDV strain X818; located in the E1 coding region; sense primer; M: A or C), OL BDV3660R (5′-TCTTKAYKGGCTCATCCTTC-3′, corresponding to nucleotides 3660–3679 of the genomic sequence of BDV strain X818; located in the p7 coding region; antisense primer; K: G or T, Y: C or T), and OL G3850R (5′-CCTAGTGTTGTGAGGAAG-3′, corresponding to nucleotides 3817–3834 of the complete genomic sequence of pestivirus strain H138; located in the NS2 coding region; antisense primer) (Avalos-Ramirez et al., 2001; Becher, Orlich, and Thiel, 1998).

### *Reverse transcription–polymerase chain reaction and cloning of $N^{pro}$ and E2 genes*

RNA preparation, RT, and PCR were done as described previously (Becher et al., 1997). Part of the 5′ NTR and the genomic region encoding $N^{pro}$, C, and part of $E^{ms}$ were amplified by RT–PCR using primers OL 1400R and OL 100. The genomic region encoding the entire glycoprotein E2 was amplified by RT–PCR using (i) primer OL P2250 and primer OL RP7R1 (BDV-2 strains 17385, Chemnitz AZ79, 466, and BDV-3 strain Gifhorn), (ii) primer OL BDV2240 and primer OL BDV3660R (BDV-1 strains T1802 and V2536), and (iii) primer OL P2250 and primer OL G3850R (pestivirus strain PG-2). Finally, the amplified cDNA fragments were cloned into pCR2.1 (Invitrogen, De Schelp, The Netherlands).

### *Nucleotide sequencing and phylogenetic analysis*

Nucleotide sequences were determined by cycle sequencing using a Thermo Sequenase kit (Amersham Pharmacia Biotech) and the DNA sequencer Licor 4000L (MWG Biotech). All sequences were determined by sequencing three independent cDNA clones; for each pestivirus isolate the degree of identity among three clones was $>$99.5%. Computer analysis of sequence data was performed by using HUSAR (DKFZ, Heidelberg, Germany), which provides the GCG (Devereux, Haeberli, and Smithies, 1984) and PHYLIP software packages (Felsenstein, 1985, 1993). Multiple sequence alignments of the nucleotide and deduced amino acid sequences were generated with programs PILEUP and CLUSTAL. Phylogenetic trees were constructed using the neighbor-joining method. The robustness of the phylogenetic analysis and significance of the branching order were determined by bootstrap analysis carried out on 1000 replicates using PHYLIP programs SEQBOOT and CONSENSE (Felsenstein, 1985, 1993; Hedges, 1992). Evolutionary distances between sequences were es-

timated by using the Kimura 2-parameter method (Kimura, 1980).

### Antisera

Antisera against BDV-1 strain V2536, BDV-2 strain 17385, and BDV-3 strain Gifhorn were produced in 4- to 6-month-old sheep after inoculation (intranasal and intramuscular) with $1 \times 10^7$ $TCID_{50}$ of the respective pestivirus strain. Blood samples were collected at 6 weeks p.i.. The antisera raised against BVDV-1 strains NY-1 and C86, BVDV-2 strains Gi-2 and 890, BDV-1 strains X818 and L83/84, CSFV strains Italy and Pader as well as against pestivirus strains H138 and V60 have been described previously (Avalos-Ramirez et al., 2001).

### Neutralization assay

Neutralization experiments were performed in microtiter plates as described previously (Avalos-Ramirez et al., 2001). Each assay was performed with quadruplicate samples. The titer was expressed as the reciprocal of the serum dilution that completely inhibited viral replication in 50% of the wells.

### Calculation of antigenic similarity

The serological relatedness of pestivirus strains was determined by calculation of the coefficient of antigenic similarity ($R$) between each pair of strains (Archetti and Horsfall, 1950); the following formula was used:

$$R = 100 \times \sqrt{\frac{\text{titer strain A with antiserum B} \times \text{titer strain B with antiserum A}}{\text{titer strain A with antiserum A} \times \text{titer strain B with antiserum B}}}$$

Sera with a titer $<8$ were assigned a titer of 1.

### Nucleotide sequence accession numbers

Sequence data from this study have been deposited with the EMBL and GenBank data libraries under accession nos. AY163647–AY163660 (Table 1).

## Acknowledgments

We thank U. Schaarschmidt (Landesuntersuchungsamt für das Gesundheits- und Veterinärwesen Sachsen, Chemnitz, Germany) and O.C. Straub (BFAV, Tübingen, Germany) for providing the ovine pestivirus isolates Chemnitz AZ 79 and 466, respectively. Ramiro Avalos-Ramirez was supported by a doctoral research fellowship from Secretaría de Educación Pública and Universidad Autonoma de Nuevo León, Mexico (PROMEP-UANL). This study was supported by Intervet International BV (Project 75/73.1808.720).

## References

Archetti, I., Horsfall, F.L., 1950. Persistent antigenic variation of influenza A viruses after incomplete neutralization in ovo with heterologous immune serum. J. Exp. Med. 92, 441–462.

Avalos-Ramirez, R., Orlich, M., Thiel, H.-J., Becher, P., 2001. Evidence for the presence of two novel pestivirus species. Virology 286, 456–465.

Baker, J.C., 1987. Bovine viral diarrhea virus: a review. J. Am. Vet. Med. Assoc. 190, 1449–1458.

Becher, P., König, M., Paton, D., Thiel, H.-J., 1995. Further characterization of border disease virus isolates: evidence for the presence of more than three species within the genus pestivirus. Virology 209, 200–206.

Becher, P., Meyers, G., Shannon, A.D., Thiel, H.-J., 1996. Cytopathogenicity of border disease virus is correlated with integration of cellular sequences into the viral genome. J. Virol. 70, 2992–2998.

Becher, P., Orlich, M., Kosmidou, A., König, M., Baroth, M., Thiel, H.-J., 1999. Genetic diversity of pestiviruses: identification of novel groups and implications for classification. Virology 262, 64–71.

Becher, P., Orlich, M., Shannon, A.D., Horner, G., König, M., Thiel, H.-J., 1997. Phylogenetic analysis of pestiviruses from domestic and wild ruminants. J. Gen. Virol. 78, 1357–1366.

Becher, P., Orlich, M., Thiel, H.-J., 1998. Complete genomic sequence of border disease virus, a pestivirus from sheep. J. Virol. 72, 5165–5173.

Becher, P., Shannon, A.D., Tautz, N., Thiel, H.-J., 1994. Molecular characterization of border disease virus, a pestivirus from sheep. Virology 198, 542–551.

Becher, P., Thiel, H.-J., 2002. Genus *Pestivirus (Flaviviridae)*, in: Tidona, C.A., Darai, G. (Eds.), The Springer Index of Viruses. Springer-Verlag, Heidelberg, Germany, pp. 327–331.

Cay, B., Chappuis, G., Coulibaly, C., Dinter, Z., Edwards, S., Greiser-Wilke, I., Gunn, M., Have, P., Hess, G., Juntti, N., Liess, B., Mateo, A., Mc Hugh, P., Moennig, V., Nettleton, P., Wensvoort, G., 1989. Comparative analysis of monoclonal antibodies against pestiviruses: report of an international workshop. Vet. Microbiol. 20, 123–129.

Collett, M.S., Larson, R., Gold, C., Strick, D., Anderson, D.K., Purchio, A.F., 1988. Molecular cloning and nucleotide sequence of the pestivirus bovine viral diarrhea virus. Virology 165, 191–199.

Couvreur, B., Letellier, C., Collard, A., Quenon, P., Dehan, P., Hamers, C., Pastoret, P.-P., Kerkhofs, P., 2002. Genetic and antigenic variability in bovine viral diarrhea virus (BVDV) isolates from Belgium. Virus Res. 85, 17–28.

Dekker, A., Wensvoort, G., Terpstra, C., 1995. Six antigenic groups within the genus pestivirus as identified by cross neutralization assays. Vet. Mic. 47, 317–329.

Devereux, J., Haeberli, P., Smithies, O.A., 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12 (1), 387–395.

Doyle, L.G., Heuschele, W.P., 1983. Bovine viral diarrhea virus infection in captive exotic ruminants. J. Am. Vet. Med. Assoc. 183, 1257–1259.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Felsenstein, J., 1993. PHYLIP (Phylogeny Inference Package) 3.5c: Department of Genetics, University of Washington, Seattle.

Hamblin, C., Hedger, R.S., 1979. The prevalence of antibodies to bovine viral diarrhea/mucosal disease virus in African wildlife. Comp. Immunol. Microbiol. Infect. Dis. 2, 295–303.

Hedges, S.B., 1992. The number of replications needed for accurate estimations of the bootstrap P value in phylogenetic studies. Mol. Biol. Evol. 9, 366–369.

Heinz, F.X., Collett, M.S., Purcell, R.H., Gould, E.A., Howard, C.R., Houghton, M., Moormann, R.J.M., Rice, C.M., Thiel, H.-J., 2000.

Family Flaviviridae, in: van Regenmortel, C.M.F.M.H.V., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R., Wickner, R.B. (Eds.), Virus Taxonomy: Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, CA, pp. 859–878.

Howard, C.J., Brownlie, J., Clarke, M.C., 1987. Comparison by the neutralisation assay of pairs of non-cytopathogenic and cytopathogenic strains of bovine virus diarrhoea virus isolated from cases of mucosal disease. Vet. Microbiol. 19, 13–21.

Hubalek, Z., 1982. Numerical comparative serology—the methods. J. Appl. Bacteriol. 52, 307–318.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.

Meyers, G., Rümenapf, T., Thiel, H.-J., 1989. Molecular cloning and nucleotide sequence of the genome of hog cholera virus. Virology 171, 555–567.

Nagai, M., Ito, T., Sugita, S., Genno, A., Takeuchi, K., Ozawa, T., Sakoda, Y., Nishimor, T., Takamura, K., Akashi, H., 2001. Genomic and serological diversity of bovine viral diarrhea virus in Japan. Arch. Virol. 146, 685–696.

Nettleton, P.F., 1990. Pestivirus infections in ruminants other than cattle. Rev. Sci. Tech. Off. Int. Epizoot. 9, 131–150.

Paton, D.J., Sands, J.J., Lowings, J.P., Smith, J.E., Ibata, G., Edwards, S., 1995. A proposed division of the pestivirus genus using monoclonal antibodies, supported by cross-neutralisation assays and genetic sequencing. Vet. Res. 26 (2), 92–109.

Pellerin, C., Van Den Hurk, J., Lecomte, J., Tijssen, P., 1994. Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virology 203, 260–268.

Plowright, W., 1969. Joint Campaign Against Rinderpest. First Technical Review Meeting, Phase IV, Mogadiscio, Kenya.

Ridpath, F.F., Bolin, S.R., Dubovi, E.J., 1994. Segregation of bovine viral diarrhea virus into genotypes. Virology 205, 66–74.

Ridpath, J.F., Bolin, S.R., 1995. The genomic sequence of a virulent bovine viral diarrhea virus (BVDV) from the type 2 genotype: detection of a large genomic insertion in a noncytopathic BVDV. Virology 212, 39–46.

Roehe, P.M., Woodward, M.J., Edwards, S., 1992. Characterization of p20 gene sequences from a border disease-like pestivirus isolated from pigs. Vet. Microbiol. 33, 231–238.

Schaarschmidt, U., Schirrmeier, H., Strebelow, G., Wolf, G., 2000. Detection of Border Disease in a sheep flock in Saxonia. Berl. Münch. Tierärztl. Wschr. 113, 284–288.

Sullivan, D.G., Chang, G.-J., Trent, D.W., Akkina, R.K., 1994. Nucleotide sequence analysis of the structural gene coding region of the pestivirus border disease virus. Virus Res. 33, 219–228.

Thiel, H.-J., Plagemann, P.G.W., Moenning, V., 1996. Pestiviruses, in: Fields, B.N., Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, 3rd ed., Vol. 1. Lippincott–Raven, Philadelphia, PA, pp. 1059–1073.

Vilcek, I., Paton, D.J., Durkovic, B., Strojny, L., G., I., Moussa, A., Loitsch, A., Rossmanith, W., Vega, S., Scicluna, M.T., Palfi, V., 2001. Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. Arch. Virol. 146, 99–115.

Vilcek, S., Nettleton, P.F., Paton, D.J., Belak, S., 1997. Molecular characterization of ovine pestiviruses. J. Gen. Virol. 78, 725–735.

Waldvogel, A.S., Ehrensperger, F., Straub, O.C., Pospischil, A., 1995. An immunohistochemical study of the distribution of border disease virus in persistently infected sheep. J. Comp. Pathol. 113, 191–200.