Genetic and antigenic characterization of an atypical pestivirus isolate, a putative member of a novel pestivirus species

Horst Schirrmeier, Günther Strebelow, Klaus Depner, Bernd Hoffmann and Martin Beer

Friedrich-Loeffler-Institut, Institute of Diagnostic Virology, Boddenblick 5a, 17493 Greifswald-Insel Riems, Germany

The genus Pestivirus within the family Flaviviridae currently consists of four different main species: Classical swine fever virus, Bovine viral diarrhea virus types 1 and 2 and Border disease virus. A fifth tentative species is represented by an isolate from a giraffe. In this study, a completely new pestivirus, isolated from a batch of fetal calf serum that was collected in Brazil, is described. It is proposed that the isolate D32/00_'HoBi' may constitute a novel sixth pestivirus species, because it is genetically, as well as antigenically, markedly different from all other pestiviruses. Based on the entire Npro- and E2-encoding sequences, identities of < 70 % to all other pestivirus species were determined. Similarly, cross-neutralization and binding studies using antisera and mAbs revealed marked antigenic differences between D32/00_'HoBi' and all other pestiviruses.

The genus Pestivirus belongs to the family Flaviviridae and consists of four separate virus species: Bovine viral diarrhea virus types 1 and 2 (BVDV-1 and -2), Classical swine fever virus (CSFV) and Border disease virus (BDV). A tentative fifth species is defined by an atypical pestivirus that was isolated from a giraffe (van Regenmortel et al., 2000). All pestiviruses are genetically and structurally related and serological cross-reactivity between all members of the genus has been reported. The natural hosts of pestiviruses are domestic and wild ruminants, as well as porcines (Thiel et al., 1996). The host ranges of BVDV and BDV are not restricted to cattle and sheep, but include a wide range of hosts in the Artiodactyla (Plowright, 1969; Hamblin & Hedger, 1979; Doyle & Heuschele, 1983; Nettleton, 1990; Becher et al., 1997), whereas CSFV has only been isolated from pigs (Dahle et al., 1987; Thiel et al., 1996). The pestivirus virion is enveloped and contains a genome that consists of a single-stranded RNA of approximately 12.5 kb and positive polarity (Collett et al., 1988; Meyers et al., 1989; Ridpath & Bolin, 1995; Becher et al., 1998). It contains a single ORF that is flanked by 5' and 3' untranslated regions (UTRs) and encodes the structural proteins capsid, EKNNS, E1 and E2, as well as the non-structural proteins Npro, p7, NS2–3 (NS2, NS3), NS4A, NS4B, NS5A and NS5B (Donis, 1995). For genetic analyses, the 5'-UTR (Vilček et al., 1994; Wolfmeyer et al., 1997; Beer et al., 2002) and the regions encoding Npro (Becher et al., 1997, 2003), E2 (van Rijn et al., 1997) and NS3 (Ridpath et al., 1994; Pellerin et al., 1995) have primarily been utilized and the Npro-encoding region is thought to yield a precise distinction of different pestivirus types and subtypes (Becher et al., 1997, 2003, Avalos-Ramirez et al., 2001). However, the highly conserved 5'-UTR gives nearly identical results, particularly concerning the allocation of pestivirus isolates to species or genotypes (Vilček et al., 2001). Important criteria for the classification of pestiviruses are their similarity at the nucleotide level, as well as their reactivity in binding assays with mAbs and cross-neutralization tests with homologous and heterologous polyclonal antisera. By using these techniques, the giraffe isolate was shown to differ clearly from all other known pestiviruses (Harasawa et al., 2000; Avalos-Ramirez et al., 2001), leading to a new, tentative pestivirus species, Pestivirus of giraffe (van Regenmortel et al., 2000). In addition, new and previously unclassified pestiviruses from reindeer (Reindeer-1), pig (Gifhorn) and sheep (Stolpe, Chemnitz) have recently been demonstrated to form distinct genotypes within BDV species (BDV-2 and -3) (Becher et al., 2003; H. Schirrmeier, K. Depner, G. Strebelow & M. Beer, unpublished data). In this study, we report the genetic and antigenic characterization of a new atypical pestivirus that was isolated from a batch of fetal calf serum (FCS) originating from Brazil. The marked differences of the investigated virus isolate from other pestiviruses led to the conclusion that the virus strain designated D32/00_'HoBi' may be a member of a novel pestivirus species.

The GenBank/EMBL/DDBJ accession numbers for the 5'-UTR, Npro, E2, NS3 and 3'-UTR sequences of isolate D32/00_'HoBi' reported in this paper are AY489116, AY489117, AY604725, AY713481 and AY604726, respectively.
D32/00 _HoBi_ was isolated from fetal sheep thymus cells ‘SFT-R’ [RIE43, Collection of Cell Lines in Veterinary Medicine (CCLV), Insel Riems] that were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS batch 547 from Brazil (Biochrom). PCR analysis directly from the FCS sample, as well as inoculation of KOP-R cells, a diploid bovine oesophageal cell line (RIE244, CCLV), demonstrated clearly that this particular batch (547) was the source of contamination with D32/00 _HoBi_. Most efficient replication of the virus was observed with bovine cell lines. In contrast, no efficient virus propagation was detectable after inoculation of porcine kidney PK-15 cells (data not shown). D32/00 _HoBi_–infected KOP-R cells were tested in binding assays with a panel of pestivirus-specific mAbs directed against NS2–3, E RNS and E2 (Table 1; Peters et al., 1986; Moennig et al., 1987; Edwards et al., 1988, 1991; Paton et al., 1994, 1995). Immunofluorescence (IF) and immunoperoxidase analyses were performed as described previously (Depner et al., 2001). It could be demonstrated that three of the five NS2–3-specific mAbs (C16, 103/105 and 435) were able to detect D32/00 _HoBi_–infected cells (Table 1). Interestingly, the BVDV-1- and BDV-specific mAb 160 (Beer & Wolf, 1999) was unable to bind, and only mAbs 103/105 and C16 reacted with all strains tested (Table 1). In addition, only two (433 and 434) of the eight E RNS -specific mAbs tested, which normally detect BVDV-2 isolates, and none of the ten E2-specific mAbs reacted with D32/00 _HoBi_ in the binding assay (Table 1). This markedly restricted reaction pattern of the newly isolated virus was significantly different from those of other pestiviruses. However, failure of mAb 160 to bind to D32/00 _HoBi_–infected cells and interaction with the E RNS -specific mAbs 433 and 434 are related most closely to the reaction pattern of BVDV-2 and the Giraffe-1 strains (Table 1).

For sequencing, RNA was isolated from inoculated KOP-R cell cultures and directly from contaminated FCS by using TRIzol reagent (Invitrogen). Different parts of the D32/00 _HoBi_ genome were amplified by RT-PCR as described previously (Pfeffer et al., 2000; Schaarschmidt et al., 2000). For amplification of a 5′-UTR fragment and the entire Npro-encoding region, the primer pairs BVD_II (sense; 5′-GGTAGCAACAGTGGTGAGTTC-3′)/BVD_II_NTR51 (antisense; 5′-CAACTCCATGTGCCATGTAC-3′) and PP235F (sense; 5′-AYGTGGACGAGGGCRYGCCCA-3′)/PP1040R (antisense; 5′-CCYTTCTTYYTNACCTGGTA-3′) were used. Amplification products generated with both primer pairs cover a genomic region that corresponds to nt 139–1059 of BVDV-1 strain NADL (Collett et al., 1988; GenBank accession no. NC_001461). In addition, the entire E2-encoding sequence and a 135 bp fragment of the 3′-UTR were amplified by using the primer pairs Hobi1300-F (sense; 5′-CAGGAGCTGAGGACCTTAG-3′)/PP3450-R (antisense; 5′-TTBARCATGATTGYTG-GAAGTA-3′), Hobi 3400-F (sense; 5′-CTTCAACTGGACTGAGGCG-3′)/PP4600-R (antisense; 5′-AYCTCTTCTATTTACTACGGA-3′) and PP3′-1F (sense; Table 1. Reactivity of NS2–3-, E RNS - and E2-specific mAbs with members of different pestivirus species

<table>
<thead>
<tr>
<th>Species (strain)</th>
<th>Anti-NS2–3 mAbs</th>
<th>Anti-E RNS mAbs</th>
<th>Anti-E2 mAbs</th>
</tr>
</thead>
<tbody>
<tr>
<td>BVDV-1 (NADL)</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BVDV-1 (CS0064)</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>BVDV-2 (Moredun)</td>
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<td>++</td>
</tr>
<tr>
<td>BDV-2 (Reindeer-1)</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>BDV-3 (Gifhorn)</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CSFV (Alfort187)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pestivirus of giraffe (Giraffe-1)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D32/00 <em>HoBi</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
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*Commercially available (c.c.pro, Neustadt, Germany). **Kindly provided by I. Greiser-Wilke (TIHo, Hannover, Germany) (Peters et al., 1986). §Kindly provided by T. Sandvik (CVL, Weybridge, UK) (Edwards et al., 1991; Paton et al., 1994). ¶Kindly provided by E. Weiland (BFAV, Tübingen, Germany) (Weiland et al., 1990).
The RT-PCR products were cloned directly and three independent plasmid clones were sequenced by using M13 universal and reverse primers. Subsequently, a consensus sequence of the amplified and cloned fragments was generated. Part of the 5’-UTR (184 bp fragment; GenBank accession no. AY489116), the entire Npro-encoding sequence was amplified by using the primer pairs Hobi 4920-F (sense; 5’-GAGGGCATATGCATGGGGA-3’)/PP6200-R (antisense; 5’-GGGCTATGAACCTCTCTAT-3’) and Hobi 6120-F (sense; 5’-AGCTGGGTCGGT-CACAAAC-3’)/PP7200-R (antisense; 5’-AACTGKARGTG-DGTKGTGTC-3’).

The results of the cross-neutralization assays corroborated the classification of D32/00_‘HoBi’ as the putative prototype of a novel species within the genus Pestivirus. By using well-defined reference sera from pigs (serum against CSFV strain 976/43; kindly provided by the EU reference laboratory for CSFV, TiHo Hannover, Germany) or rabbit hyperimmune sera (sera against BVDV-1 strain Paplitz, BVDV-2 strain Munich-2, BVDV-1 strain Morendon, BVDV-2 strain Reindeer-1 and BVDV-3 strain Gifhorn; H. Schirrmieer, G. Strebelow & K. Depner, unpublished data), no or only low neutralizing titres against D32/00_‘HoBi’ could be determined (Fig. 1d). No neutralizing activity versus D32/00_‘HoBi’ was detectable with four of the six sera tested (neutralizing antibody titre < 1:5), although homologous titres varied from 1:120 to 1:1280 (Fig. 1d). A D32/00_‘HoBi’-specific immune serum obtained from an inoculated calf with a homologous titre of > 1:640 was used as a positive control (Fig. 1d). With this antiserum, a heterologous titre of 1: 40 could be determined against the giraffe strain (data not shown).

As D32/00_‘HoBi’ was isolated from an FCS sample, its real host remains unidentified. Nevertheless, domestic or free-ranging ruminants, particularly of the genus Bos within the family Bovidae, are the most likely target species of D32/00_‘HoBi’. In a first animal experiment, pigs (n = 2) and cattle (n = 2) were infected intranasally with 2 x 10^6 TCID50 D32/00_‘HoBi’ per animal. No D32/00_‘HoBi’ virus could be reisolated from the inoculated pigs, none of the contact pigs seroconverted and no clinical symptoms or leukopenia could be detected. However, both inoculated pigs seroconverted versus D32/00_‘HoBi’.

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In contrast, viraemia was detected at day 5 post-infection in the infected calves, D32/00_‘HoBi’ virus was shed for several days (days 3–6 post-infection) and slight leukopenia, as well as a mild increase in body temperature, could be detected (data not shown). As observed for the inoculated pigs, the infected calves did not show clinical signs during the whole experiment.

Taken together, comparative sequence analyses, binding studies with mAbs and cross-neutralization assays revealed marked differences between D32/00_‘HoBi’ and all current pestivirus species. We propose that the low nucleotide sequence identities between D32/00_‘HoBi’ and selected members of all other species based on the 5’-UTR, Npro, E2 and 3’-UTR sequences support classification as a novel pestivirus species; the low sequence similarities are consistent with previous values that have been used for division into species (Vilček et al., 1994; Becher et al., 1997, 2003; Avalos-Ramirez et al., 2001). Thus, D32/00_‘HoBi’ may be classified as a representative of a novel tentative species within the genus Pestivirus by analogy to the species Pestivirus of giraffe (van Regenmortel et al., 2000). In addition, the NS3 nucleotide sequence comparison generated the same identity values between D32/00_‘HoBi’ and all other pestivirus species. Interestingly, the tree based on the NS3 sequences revealed a slightly enhanced phylogenetic
characterization of an atypical pestivirus isolate

Fig. 1. (a–c) Phylogenetic trees generated from the entire N-pro-, E2- and NS3-encoding nucleotide sequences of selected members of known species of the genus Pestivirus and D32/00_'HoBi'. The sequences of the virus strains Osloss, NADL, PT810, Giessen-6, 890, Alfort187, Brescia, CSFV39, X818, Gilhorn, Reindeer-1 and Giraffe-1 were taken from GenBank (accession nos M96687, NC_001461, AY078406, AF144470, AF144612, U18059, X87939, AF091661, AF407399, NC_003679, AY163653, AY163660, NC_003677 and NC_003678). The N-pro, E2 and NS3 sequences of D32/00_'HoBi' were generated in this study. The trees were constructed by using the software PUZZLE (Strimmer & von Haeseler, 1996) and TREEVIEW (Page, 1996). The numbers refer to the percentage support for the internal branches of the quartet-puzzling tree (1000 puzzling steps). Branch lengths are proportional to genetic distances. (d) Cross-neutralization assays of D32/00_'HoBi' with sera specific for members of the different species and major genotypes within the genus Pestivirus. D32/00_'HoBi' was not neutralized efficiently by heterologous sera (black bars), with titres ranging between <1:5 and 1:10. In contrast, homologous neutralization titres (grey bars) reached levels between 1:120 and 1:1280. Strains used for the generation of antisera are indicated.

Table 2. Percentage nucleotide identity of members of different pestivirus species or major genotypes, based on the entire N-pro sequence

<table>
<thead>
<tr>
<th>Species/genotype (strain)</th>
<th>Nucleotide identity (%) with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5 6 7 8</td>
</tr>
<tr>
<td>D32/00_'HoBi'</td>
<td>100 64 65 67 66 65 66 65</td>
</tr>
<tr>
<td>BVDV-2 (US890)</td>
<td>100 67 67 66 66 65 65 65</td>
</tr>
<tr>
<td>BVDV-1 (NADL)</td>
<td>100 66 67 67 64 64 64 68</td>
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<tr>
<td>CSFV (Alfort187)</td>
<td>100 70 69 68 67</td>
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<tr>
<td>BDV-1 (X818)</td>
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<tr>
<td>BDV-2 (Reindeer-1)</td>
<td>100 73 76</td>
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<tr>
<td>BDV-3 (Gilhorn)</td>
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<tr>
<td>Pestivirus of giraffe</td>
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</tbody>
</table>

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References


