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1 **Atypical ‘HoBi’-like pestiviruses-** recent findings and implications thereof

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13 *Keywords: Pestivirus, BVDV, HoBi, FCS, Contamination,*

14
15 **Abstract**

16 In 2004 an atypical pestivirus named D32/00_‘HoBi’, isolated from foetal calf serum (FCS)
17 originating from Brazil, was described (Schirrmeier et al., 2004). A few years later, a closely
18 related virus (Th/04_KhonKaen) was detected in serum from a calf in Thailand, indicating
19 that this group of atypical pestiviruses already is spread in cattle populations in various
20 regions of the world. At the Friedrich-Loeffler-Institute, Insel Riems, Germany, FCS batches
21 are regularly tested for pestivirus contamination, in general with positive PCR results, and in
22 some cases the contaminants have been typed as ‘HoBi’-like. At the National Veterinary
23 Institute (SVA) in Uppsala, Sweden, a recent event with contaminated FCS ruined much of
24 the ongoing cell culture work. From the FCS and the contaminated cells we were able to
25 amplify and sequence nucleic acid from three different pestivirus strains, including BVDV-1,

26 -2 and 'HoBi'-like; this in a commercial FCS that had been tested free from pestivirus by the
27 manufacturer.

28 In this short communication we review the current status of atypical 'HoBi'-like pestiviruses,
29 describe recent findings and discuss the implications thereof.

30

31 **1. Introduction**

32 Bovine Viral Diarrhoea Virus (BVDV) is the denomination of a heterogeneous group of
33 viruses in the family *Flaviviridae*, genus *Pestivirus*, which are economically important
34 pathogens that primarily infect ruminants. Infections with BVDV are endemic in cattle
35 populations in most parts of the world. The prevalence of antibody positive cattle is in general
36 high, and 1-2% of the cattle are persistently infected (PI) with the virus (Houe, 1999).

37 Because of the high prevalence of PI cattle, BVDV is also a common contaminant of FCS,
38 which is used to supplement growth medium for cell cultures for diagnostic or research
39 purposes, or for the production of biological products such as vaccines (Bolin and Ridpath,
40 1998). One possible consequence of such contamination became evident during a compulsory
41 vaccination campaign against bovine herpesvirus 1 in Dutch cattle, when virulent BVDV type
42 2 (BVDV-2) was spread with a contaminated live vaccine, causing high morbidity and
43 mortality (Barkema et al., 2001).

44 Currently there are two recognized genotypes of BVDV (BVDV-1 and -2) that together with
45 Border Disease Virus (BDV) and Classical Swine Fever Virus (CSFV) constitute the four
46 accepted species of the genus *Pestivirus*. In addition, a tentative fifth species is represented by
47 a virus isolated from a giraffe (Becher et al., 2003). The pestivirus genome consists of single
48 stranded positive-sense RNA of approximately 12.3 kb., with one open reading frame flanked
49 by 5' and 3' untranslated regions (UTR). Classification of pestiviruses is based on the genetic
50 relatedness between isolates, including genetic similarity with the type virus of the species

51 (Heinz et al., 2000). Furthermore, because pestiviruses are serologically cross-reactive,
52 species demarcation considers antigenic relationships based on binding assays with
53 monoclonal antibodies or cross-neutralisation assays with polyclonal antisera (Hamers et al.,
54 2001; Becher et al., 2003).

55

56 **2. Atypical ‘HoBi’-like pestiviruses**

57 In 2004 an atypical pestivirus named D32/00_‘HoBi’ was isolated from FCS originating from
58 Brazil (Schirrmeyer et al., 2004). Due to the genetic and antigenic properties that markedly
59 differed from previously described pestivirus species, this virus was proposed as a
60 representative of a possible novel species. This was the first of several reports describing
61 more or less closely related atypical pestiviruses: Stalder et al. (2005) described ‘HoBi’-like
62 viruses isolated from contaminated cell culture (probably originating from a batch of FCS
63 produced in South America) and from a Brazilian buffalo; Cortez et al. (2006) detected
64 ‘HoBi’-like viruses in aborted Brazilian bovine fetuses; Ståhl et al. (2007) detected a related
65 virus in serum from a calf in Thailand, and Kampa et al. (2008) further described evidence of
66 exposure to ‘HoBi’-like viruses in additional cattle herds in Thailand. In sum, these reports
67 suggested that ‘HoBi’-like pestiviruses already were spread in cattle populations, particularly
68 in Brazil, but also outside South America. Whereas the main concern caused by the detection
69 of ‘HoBi’-like viruses in FCS focused on issues related to the biosafety of biological products
70 of bovine origin, the isolation of ‘HoBi’-like pestiviruses from aborted foetuses also
71 indicated direct clinical implications (Cortez et al., 2006).

72

73 **3. Recent findings at FLI and SVA**

74 Since pestivirus contamination of FCS is a permanent problem in cell culture laboratories, the
75 virological institutes are regularly screening serum batches for the presence of these viral

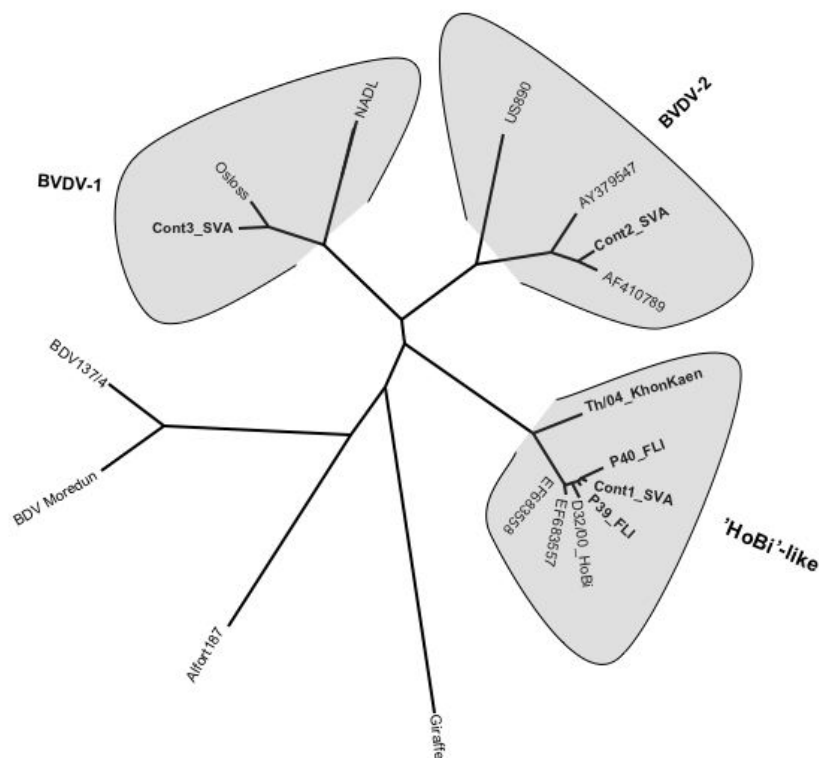
76 agents. The screening is mainly focusing on the presence of BVDV-1 and -2. However,
77 recently the contamination with atypical pestiviruses is also a question of increasing
78 importance. At the Friedrich-Loeffler-Institute a recent investigation of 25 batches was made
79 using virus isolation on calf oesopharynx cells (KOP-R, L244, Collection of Cell Lines in
80 Veterinary Medicine (CCLV), Insel Riems), and conventional and real-time RT-PCR
81 protocols for the amplification of the 5' UTR (Vilcek et al., 1994; Gaede et al., 2005;
82 Hoffmann et al., 2006), in some cases followed by typing of the contaminating viruses by
83 sequencing of the amplicons. In all but one batch, traces of pestivirus nucleic acids were
84 detected, demonstrating pestivirus contamination. In two of the batches, the contaminating
85 virus was typed as 'HoBi'-like. These 'HoBi'-like viruses were only detected by the real-time
86 RT-PCR assays and not by the conventional RT-PCR protocol based on the commonly used
87 324/326 primer pair, a finding that is in accordance with previous observations (Schirrmeier
88 et al., 2004). Furthermore, BVDV type 1 was isolated from three of the contaminated batches;
89 the other virus isolation attempts remained negative also after three passages.

90 At SVA bovine turbinate (BT) cells are used for virus isolation within the Swedish BVD
91 control and eradication programme. For almost 20 years pestivirus-free FCS was produced in-
92 house, but since the last few years, however, commercial FCS is being used for the cell
93 cultures. Although all commercial FCS is being tested for pestivirus contamination, a recent
94 event demonstrated the vulnerability of the system. A new batch of FCS of Brazilian origin
95 had been used for the BT cells between passages 4 and 9 when suddenly all virus isolations
96 turned positive for BVDV. Further investigations demonstrated contamination also in other
97 cell cultures where the FCS had been used, and the FCS was considered the most likely
98 source. From the FCS and the contaminated cells, the 5' UTR from three different pestivirus
99 strains, including BVDV-1, -2 and 'HoBi'-like, could be amplified and sequenced (Figure 1).

100

101 **4. Genetic diversity**

102 To date at least nine atypical ‘HoBi’-like pestiviruses have been described; eight of known or
 103 probable Brazilian origin, and one of Thai origin. Phylogenetic analysis based on the longest
 104 available overlapping segment of the 5’UTR (approximately 140 nucleotides) demonstrates
 105 genetic diversity within the group and suggests that two subgroups can be defined: one
 106 represented by D32/00_‘HoBi’ and the seven other ‘HoBi’-like viruses of Brazilian origin,
 107 and the other by Th04_KhonKaen (Figure 1). The sequence identity within the partial 5’UTR
 108 used for the analysis ranges between 90 and 93% between the subgroups, and between 96 and
 109 99% within the ‘HoBi’ subgroup.



110

111 **Figure 1.** Unrooted Neighbor-joining phylogram generated from a 140 nucleotides fragment of the 5’ UTR
 112 sequences of pestivirus contaminants detected in FCS at FLI and SVA (in bold), and representatives of each
 113 known species within the genus *Pestivirus*, including the tentative pestivirus of giraffe and the atypical ‘HoBi’-
 114 like pestiviruses. The sequences of the reference strains NADL, Osloss, US890, Giraffe H138, Alfort187,
 115 BDV137/4, Moredun, D32/00_‘HoBi’, the previously described Th/04_KhonKaen and the Brazilian strains

116 AY379547, AF410789, EF683558 and EF683557 were obtained from the GenBank (accession nos. NC001461,
117 M96687, Z79772, AB040131, X87939, U65052, U65022, AY489116, DQ897641, AY379547, AF410789,
118 EF683558 and EF683557).

119

120 **5. Discussion**

121 The growing number of reports on 'HoBi'-like pestiviruses, including the recent findings
122 reported herein, raises two main concerns. The first is related to the general problem of
123 pestivirus contamination of FCS with implications for the biosafety of biological products
124 that contain FCS, such as vaccines, diagnostic reagents and kits, stem cells and other products
125 of the bioindustry. The second is related to the emergence and spread of atypical pestiviruses,
126 possibly via biological products (such as vaccines), with possible implications for animal
127 health and disease control. Both issues have to be seriously considered at a global perspective.

128 The first issue is well known and has been addressed in several recent publications (Audet et
129 al., 2000; Studer et al., 2002; Makoschey et al., 2003), and was also the main topic during an
130 international workshop organized by the EDQM in Paris 2001 (EDQM, 2001). The European
131 Committee for Medicinal Products for Veterinary Use (CVMP) prescribes inactivation, such
132 as gamma irradiation, of FCS used in the production immunological medicinal products such
133 as vaccines, and testing for BVDV before and after the inactivation process using virus
134 isolation on bovine cells sensitive to Pestiviruses (three passages recommended; see
135 EMEA/CVMP/743/00-Rev-2, <http://www.emea.europa.eu/pdfs/vet/iwp/074300en.pdf>). Given
136 that the inactivation process is properly validated and properly carried out, this should ensure
137 a safe end product. However, it is not always evident that the inactivation process is
138 efficiently applied, as shown by the contaminated herpesvirus vaccine in the Netherlands
139 (Barkema et al., 2001), and even very low levels of contamination may have severe
140 consequences. And although testing is carried out after the inactivation in accordance with the

141 CVMP guidelines, a low-level pestivirus contamination may be missed, as shown by the
142 recent experience at SVA where the contaminating virus was not detected until after five
143 passages. Moreover, the legislation as well as the commonly used diagnostic methods are
144 optimized to ensure freedom from BVDV-1 and -2, and might not be optimal for the atypical
145 bovine pestiviruses, and as already mentioned this is particularly true for commonly applied
146 RT-PCR protocols.

147 Since the first description of D32/00_ 'HoBi', eight other related atypical pestivirus strains
148 have been detected and/or isolated seemingly independently of one another. Together with the
149 phylogenetic data presented herein that, albeit based on a short fragment of the genome,
150 demonstrate genetic diversity and indicate subgrouping, this suggests that atypical 'HoBi'-
151 like pestiviruses are not recently emerged viruses of cattle, but rather that they have existed
152 long enough in the cattle population to spread and evolve. The observation that the only virus
153 detected outside of the South American continent, i.e. Th/04_KhonKaen, is the most
154 divergent member of the group and forms a possible subgroup, is interesting. However,
155 whether this subgrouping correlates with geographical origin, or not, cannot be concluded at
156 this stage, but requires further studies.

157

158 **6. Conflicts of interest**

159 None

160

161 **7. References**

162

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