



HAL
open science

Virus recovery and full-length sequence analysis of atypical bovine pestivirus Th/04_KhonKaen

Lihong Liu, Jaruwat Kampa, Sándor Belák, Claudia Baule

► **To cite this version:**

Lihong Liu, Jaruwat Kampa, Sándor Belák, Claudia Baule. Virus recovery and full-length sequence analysis of atypical bovine pestivirus Th/04_KhonKaen. *Veterinary Microbiology*, 2009, 138 (1-2), pp.62. 10.1016/j.vetmic.2009.03.006 . hal-00490550

HAL Id: hal-00490550

<https://hal.science/hal-00490550>

Submitted on 9 Jun 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: Virus recovery and full-length sequence analysis of atypical bovine pestivirus Th/04_KhonKaen

Authors: Lihong Liu, Jaruwan Kampa, Sándor Belák, Claudia Baule



PII: S0378-1135(09)00110-2
DOI: doi:10.1016/j.vetmic.2009.03.006
Reference: VETMIC 4376

To appear in: *VETMIC*

Received date: 16-9-2008
Revised date: 11-2-2009
Accepted date: 2-3-2009

Please cite this article as: Liu, L., Kampa, J., Belák, S., Baule, C., Virus recovery and full-length sequence analysis of atypical bovine pestivirus Th/04_KhonKaen, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2009.03.006

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20

**Virus recovery and full-length sequence analysis of atypical
bovine pestivirus Th/04_KhonKaen¹**

Lihong Liu^{a,b}, Jaruwan Kampa^c, Sándor Belák^{a,b}, Claudia Baule^{b,*}

*^aDepartment of Biomedical Sciences and Veterinary Public Health, Swedish University of
Agricultural Sciences, SE-751 89 Uppsala, Sweden*

*^bJoint R&D Division of Virology, The National Veterinary Institute & The Swedish
University of Agricultural Sciences, SE-751 89 Uppsala, Sweden*

^cFaculty of Veterinary Medicine, Khon Kaen University, 40002, Khon Kaen, Thailand

*Corresponding author: Mailing address: Joint R&D Division of Virology, The National
Veterinary Institute & The Swedish University of Agricultural Sciences, SE-751 89
Uppsala, Sweden. Phone: +46-18-674638. Fax: +46-18-674669.

Email: Claudia.Baule@bvf.slu.se

¹ The full-length sequence of the Th/04_KhonKaen virus has been deposited in GenBank under accession number FJ040215.

21 **Abstract**

22 Phylogenetic analysis of recently identified bovine “atypical” pestiviruses, performed
23 based on different gene regions, has revealed unclear relationships with other established
24 species, therefore, their phylogenetic position could not be determined so far. In this
25 study, the atypical pestivirus Th/04_KhonKaen was recovered from serum of a naturally
26 infected calf and the complete genome sequence was determined and analysed, as means
27 to define its position. The viral genome is 12 337 nucleotides (nt) long, and comprises a 5'
28 UTR of 383 nt, a 3' UTR of 254 nt and an open reading frame of 11700 nt, without
29 duplication of viral sequences or insertions of cellular sequences. The phylogenetic
30 analyses of the full-length sequence, performed by Neighbor-joining, maximum
31 likelihood, and the Bayesian approach, unanimously placed Th/04_KhonKaen in a single
32 lineage, distinct from the established pestivirus species, and close to bovine viral diarrhea
33 virus types 1 and 2. Furthermore, Th/04_KhonKaen and two previously reported atypical
34 pestiviruses D32/00_‘HoBi’ and CH-KaHo/cont formed a well-supported monophyletic
35 clade in trees based on the complete N^{pro} and E2 gene regions. The finding provides
36 conclusive classification of the Th/04_KhonKaen virus and confirms the standing of the
37 bovine “atypical” pestiviruses as a novel pestivirus species.

38

39

40

41

42 **Keywords:** BVDV, genotyping, pestivirus, phylogeny, Th/04_KhonKaen

43

44 1. Introduction

45

46 The pestivirus genome is a single-stranded, positive-sense RNA molecule, which
47 comprises two untranslated regions (UTRs) at the 5' and 3' ends, and one open reading
48 frame (ORF) encoding a large polyprotein. This polyprotein is co- and post-translationally
49 processed into 11-12 polypeptides in the following order: N-terminal autoprotease (N^{pro}),
50 capsid protein (C), envelope proteins (E^{ms}, E1, and E2), p7, and non-structural (NS)
51 proteins [(NS2-3 (or NS2 and NS3), NS4A, NS4B, NS5A, and NS5B)] (reviewed by
52 Rumenapf and Thiel, 2008). Recombination or point mutations in the viral genome have
53 been shown to correlate with induction of cytopathogenic effects (CPE) on cell culture
54 following infection with BVDV (Thiel et al., 2006). Accordingly, two biotypes of BVDV
55 can be distinguished: the non-cytopathogenic (ncp), that replicates without cytopathic
56 effect, and cytopathogenic (cp), that leads to death of infected cells (Thiel et al., 1996).

57

58 The genus *Pestivirus* of the family *Flaviviridae* includes four recognized species: *Bovine*
59 *viral diarrhea virus 1* (BVDV-1), *Bovine viral diarrhea virus 2* (BVDV-2), *Border*
60 *disease virus* (BDV), *Classical swine fever virus* (CSFV), and a fifth tentative species
61 *Pestivirus of giraffe* (Seventh Report of the International Committee on Taxonomy of
62 Viruses; <http://www.virustaxonomyonline.com>). A recently identified new pestivirus,
63 D32/00_‘HoBi’, was isolated from a batch of fetal calf serum originating from Brazil and
64 was proposed as a sixth pestivirus species (Schirrmeyer et al., 2004). Additional atypical
65 pestiviruses of bovine-origin have also been described, including: Brz buf 9, originally
66 isolated from a buffalo in Brazil; CH-KaHo/cont, a cell culture contaminant possibly

67 originating from a batch of fetal calf serum produced in South America (Stalder et al.,
68 2005); and Th/04_KhonKaen from a naturally infected calf in Thailand (Ståhl et al.,
69 2007). All these pestiviruses were shown to be closely related to D32/00_‘HoBi’, based on
70 trees generated from the 5’UTR and N^{pro} regions, however their final phylogenetic
71 classification could not be determined.

72

73 The Th/04_KhonKaen virus was first detected by ELISA tests and preliminarily
74 characterized by virus neutralization and partial sequence analysis of the 5' UTR (Ståhl et
75 al., 2007). Further genetic characterization of this pestivirus required a virus isolate, but
76 the fact that the serum had been inactivated hampered virus isolation by conventional
77 methods. In this study, a viable Th/04_KhonKaen virus was recovered by transfection of
78 bovine turbinate cells with RNA extracted from an inactivated serum sample, and the
79 complete sequence was determined. Comparative sequence analyses show that the
80 Th/04_KhonKaen virus, together with atypical pestiviruses D32/00_‘HoBi’ and CH-
81 KaHo/cont represent a new species within the pestivirus genus.

82

83 **2. Materials and Methods**

84

85 **2.1. Virus recovery from an inactivated serum sample**

86

87 The serum sample was obtained from a calf in a herd in Thailand. According to the
88 legislation in Sweden the sample had to be heat-inactivated at 56°C for 90 min, therefore it
89 was no longer suitable for virus isolation by conventional methods. To recover virus, RNA

90 was extracted from this sample using TRIzol Reagent (Invitrogen, Carlsbad, USA), and
91 purified with phenol-chloroform. The RNA was electroporated into BVDV-free bovine
92 turbinate (BT) cells in a cell suspension containing 1×10^6 cells, using a Bio-Rad Gene
93 Pulser Xcell electroporator. The cells were grown in Eagle's minimum essential medium
94 (EMEM) containing 10% horse serum, 1% L-glutamine, and 100 U/ml of penicillin and
95 100 μ g/ml of streptomycin, at 37°C in a CO₂ incubator. Confluent cells were split and
96 grown for four days. The cells were then frozen and thawed and the lysates were clarified
97 by centrifugation. The supernatants were used to infect fresh sub-confluent BT cells, as
98 performed routinely for virus propagation. After incubation at 37°C for four days, total
99 RNA extracted from lysates of the cells was used to confirm presence of viral RNA with a
100 pan-pestivirus RT-PCR targeting the 5' UTR.

101

102 2.2. Immunoperoxidase test

103

104 The immunoperoxidase (IPX) test was performed to demonstrate presence of BVDV
105 specific antigens in cells infected with the recovered virus. Briefly, serial dilutions of the
106 supernatant from virus-infected BT cells were used to infect BT cells grown in a 96-well
107 plate. Following incubation at 37°C for 4 days, the cells were washed once with 0.01 M
108 PBS, pH 7.6, fixed with fixation fluid and dried. After re-dehydration of the cells, an anti-
109 serum directed against BVDV-1 was added to each well and the plates were incubated at
110 room temperature for 15 min. The cells were washed three times and 50 μ l of diluted
111 rabbit anti-bovine horseradish peroxidase conjugate was added, followed by incubation

112 and washing as before. For detection, 50 μ l of substrate solution (5 ml of 0.05 M sodium
113 acetate, 6 μ l of 3% H₂O₂, and 300 μ l of chromogen solution) was used.

114

115 2.3. Amplification, sequencing and analysis of the full-length genome

116

117 2.3.1. RNA extraction

118

119 When presence of virus was confirmed by RT-PCR, infected cells were washed twice with
120 cold PBS without Mg²⁺ and Ca²⁺, and lysed with a lysis buffer containing 100 mM Tris-
121 HCl, pH 8.0, 500 mM LiCl, 5 mM DTT, 10 mM EDTA, pH 8.0 and 5% Lithium
122 Duodecyl Sulphate. Total RNA was purified from the cell lysates by phenol/chloroform
123 and precipitated in an equal volume of isopropanol. After centrifugation, the RNA pellet
124 was washed once with 70% of ice-cold ethanol. The dried pellet was re-suspended in 30 μ l
125 RNase-free water.

126

127 2.3.2. Synthesis of double-stranded cDNA

128

129 SuperScript II (Invitrogen, Carlsbad, USA) was used to synthesize the first strand cDNA
130 using 2 μ M of a gene-specific primer (PAS1) in a 20- μ l volume, following conditions
131 recommended by the manufacturer. The second strand was prepared in 150- μ l reaction
132 volume, containing the 1st strand reaction, *E. coli* DNA ligase (10 U), *E. coli* DNA
133 polymerase (40 U), *E. coli* RNase H (2 U) in 1 \times second strand buffer. The reaction was
134 performed at 16°C for 2 hours. The cDNA was purified with phenol/chloroform and

135 precipitated in 95% ethanol at -20°C overnight. The cDNA pellet was washed with 75%
136 ethanol, and resuspended in 20 μl of distilled water.

137

138 2.3.3. PCR amplification

139

140 The primers were designed based on conserved sequences of established pestiviruses and
141 the sequence of the D32/00_HoBi virus. The list of primers for PCR amplification and
142 DNA sequencing is presented in Table 1. The viral genome sequence, excluding the 5' and
143 3' ends, was amplified in two overlapping fragments by PCR using primers TF1-TR1, and
144 TF2-TR2. PCR was performed in a 25- μl volume, containing 0.5 μM of forward and
145 reverse primers, 0.5 mM dNTP mix, 1.25 U of *PfuUltra* High-Fidelity or 2.5 U EXL DNA
146 polymerase (Stratagene, La Jolla, CA, WSA), 1 μl of cDNA, and 1 \times reaction buffer with
147 MgCl_2 . After initial denaturation at 92°C for 2 min, a three-step cycling of 30 cycles was
148 performed with denaturation at 92°C for 30 sec, annealing at 55°C for 30 sec, and
149 elongation at 72°C for 7 min. Following amplification, the PCR products were separated
150 on 0.8% agarose gels, and the designed band was excised from the gel and purified with
151 the Wizard SV Gel and PCR Clean-Up System (Promega Co., Madison, WI, USA).

152

153 2.3.4. Determination of the 5' and 3' ends by RNA ligase-mediated RACE

154

155 To determine the 5' and 3' ends, a GeneRacer Kit (Invitrogen, Carlsbad, USA) was used,
156 following manufacturer's instruction. In brief, the GeneRacer RNA oligo, and RNA oligo

157 2 (Table 1) were ligated to the 5' end and 3' end of the viral genome, respectively. Ten
158 units of T4 RNA ligase were used in the reactions and ligation was performed at 37°C for
159 1 h. The reactions were then treated with proteinase K, followed by phenol/chloroform
160 purification, and precipitation of the RNA in 95% ethanol in the presence of 0.1M sodium
161 acetate. The RNA was pelleted by centrifugation, washed with 70% ethanol and
162 resuspended in 30 µl of RNase-free water. The GeneRacer 5' primer from the kit and gene-
163 specific primer T5U-R1 were used to amplify the target 5' end using a one-step RT-PCR
164 kit (Qiagen, Hilden, Germany), with the following cycling profile: 50°C for 30 min, 95°C
165 for 15 min, 35 cycles of 95°C for 15 sec, 54°C for 20 sec, and 72°C for 1 min, and a final
166 extension at 72°C for 7 min. To amplify the 3' end, the gene-specific primer T3U-F1 and
167 M13 forward primer (complementary to the 5' end of the RNA oligo) were used. The same
168 reaction was performed as for amplification of the 5' end.

169
170 The purified PCR products were cloned into pCR4-TOPO (Invitrogen, Carlsbad, USA).
171 Four clones containing inserts of the 5' and 3' ends of the genome were used for DNA
172 sequencing with M13 forward and reverse primers (Invitrogen, Carlsbad, USA).

173

174 2.3.5. DNA sequencing

175

176 An ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems,
177 Foster City, CA, USA) was used for DNA sequencing. Sequencing reactions were
178 performed in a total volume of 10 µl, containing 1.5 µl of ABI 5 × sequencing buffer, 0.5
179 µM primer, 1.0 µl of BigDye, and 1.0 µl of the purified PCR product. The thermo profile

180 was 25 cycles of denaturation at 96°C for 15 sec, annealing at 50°C for 10 sec, and
181 extension at 60°C for 4 min. To remove contaminating salts and unincorporated dye
182 terminators from the sequencing reactions, the Montage SEQ96 sequencing reaction
183 cleanup kit (Millipore Co., Billerica, MA, USA) was used, following the manufacturer's
184 instructions. Capillary electrophoresis was carried out in an ABI 3100 genetic analyzer
185 (Applied Biosystems, Foster City, CA, USA). Sequences were analyzed with multiple
186 programs of the Lasergene package (DNASTAR, Inc., Madison, WI).

187

188 2.3.6. Phylogenetic analysis

189

190 For phylogenetic analysis, a total of 24 full-length genome sequences of pestiviruses were
191 retrieved from GenBank, including 9 of CSFV, 3 of BDV, 7 of BVDV-1 and 4 of BVDV-
192 2, and Pestivirus of giraffe. The GenBank accession numbers are included in Table 2.
193 Multiple sequence alignment was performed with software BioEdit version 7.0.5.2 (Hall,
194 1999), using CLUSTAL W (Thompson et al., 1994). To avoid bias due to different
195 methods, phylogenetic analysis of complete genome sequences was performed with three
196 methods: Neighbor-joining, maximum likelihood and the Bayesian approach. All gaps
197 created by insertions were deleted by the software MEGA prior to the analyses. MEGA 4
198 (Tamura et al., 2007) was used to reconstruct the Neighbor-joining tree under the
199 nucleotide substitution model of Tamura 3-parameter, and tree reliability was evaluated by
200 bootstrapping 1000 replicates. PHYML (v2.4.4) was used for phylogeny inference
201 according to maximum likelihood criterion. The analysis settings were: Base frequency
202 estimates (ML); Proportion of invariable sites (estimated); Nucleotide substitution model

203 (GTR); Number of substitution rate categories (4); Gamma distribution parameter
204 (estimated). Following tree reconstruction, 1000 non-parametric bootstrap analyses were
205 performed to test the robustness of the hypothesis. Bayesian inference analysis was
206 performed with the software MrBayes 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and
207 Huelsenbeck, 2003), as previously described (Xia et al., 2007). In brief, the model settings
208 were a Dirichlet prior for both substitution rates (Nst=6) and state frequencies (# states=4).
209 Rate variation across sites was modeled using a γ -distribution with a proportion of sites
210 invariable (rates=invgamma). The MCMC search was run with four chains for 2 000 000
211 generations, sampling the Markov chain every 1000 generations. The first 25% trees were
212 discarded as “burn-in”. Each analysis was performed three times and a representative
213 consensus tree is shown in this paper.

214

215 In order to demonstrate the relationships among these recently described atypical
216 pestiviruses, sequences of the complete N^{pro} and E2 coding regions from 64 pestiviruses,
217 corresponding to the GenBank accession numbers listed in Table 2, were analysed with
218 the Bayesian approach. The same parameters applied for the full-length phylogeny
219 analysis were used.

220

221 **3. Results**

222

223 3.1. Virus recovery

224

225 The RNA extracted from lysates of infected cells yielded a specific PCR product on
226 amplification with a pan-pestivirus RT-PCR targeting 5' UTR. The recovered virus was
227 confirmed by the IPX test that showed presence of pestivirus-specific proteins upon
228 propagation following cell transfection. The Th/04_KhonKaen virus reacted with the
229 BVDV-1 antiserum, which also recognizes BVDV-2 and D32/00_ 'HoBi'. Cytopathic
230 effect (CPE) was not observed on isolation and propagation of the virus, supporting the
231 recovery of a non-cytopathogenic pestivirus from the serum sample.

232

233 3.2. Sequence analysis of the viral genome

234

235 The full-length sequence was assembled from partial sequences obtained from the two
236 RT-PCR fragments and the 5' and 3' ends of the genome. The complete genome contains
237 12 337 nucleotides (nt), comprising 383 nt at the 5' UTR, 254 nt at the 3' UTR and an
238 ORF of 11 700 nt. The ORF involves the 11 gene regions that correspond to predicted
239 products of polyprotein processing: the pestivirus-specific N-terminal protease N^{pro} (504
240 nt), capsid (303 nt), E^{ms} (681 nt), E1 (585 nt), E2 (1119 nt), p7 (210 nt), NS2-3 (3408 nt),
241 NS4A (192 nt), NS4B (1041 nt), NS5A (1491 nt), and NS5B (2166 nt). Neither
242 duplication of viral sequences nor insertions of cellular sequences were found in the
243 genome. Comparative sequence analysis showed that this virus shares 67.3-68.3% identity
244 with other pestivirus species, with the highest identity of 68.3 to BVDV-1 and -67.9% to
245 BVDV-2, respectively (Table 3).

246

247 The polyprotein (3899 aa) was used as a query protein for searching in the Protein Data
248 Bank (PDB). The protein BLAST revealed several conserved domains, including the N^{pro}
249 endopeptidase C53 that is unique to pestiviruses; the RNase T2 in the Erns region; the
250 pestivirus NS3 polyprotein peptidase S31 and DEXH-box helicases located in NS3; and
251 RNA-dependent RNA polymerase within NS5B. Such analysis provided additional
252 support for the classification of Th/04_KhonKaen as a pestivirus.

253

254 3.3. Phylogenetic analysis of Th/04_KhonKaen sequence

255

256 Three methods, Neighbor-joining, maximum likelihood, and the Bayesian approach were
257 exploited for phylogenetic analysis. By applying the later method, we have previously
258 shown that a total evidence approach could be used for confident classification of BVDV
259 into subgroups (Xia et al., 2007). All three methods produced a single, reliable
260 phylogenetic hypothesis that was supported by significantly high bootstrap values and
261 posterior probability (Fig. 1). The established species BVDV-1, BVDV-2, BDV, CSFV
262 separated into four monophyletic clades, and the tentative pestivirus of Giraffe formed a
263 single branch. Th/04_KhonKaen also formed a single branch positioned distinctly from
264 the four established pestivirus species and from pestivirus of Giraffe, and closely related to
265 BVDV-1 and BVDV-2. The position of Th/04_KhonKaen in the consensus trees was
266 supported by bootstrap values of 99% (Neighbor-joining), 93% (ML), and posterior
267 probability of 1.00 (Bayesian approach).

268

269 To demonstrate the relationship between the Th/04_KhonKaen virus and other atypical
270 pestiviruses D32/00_‘HoBi’ and CH-KaHo/cont, single N^{pro} and E2 gene analyses were
271 performed. As shown in Fig. 2 (a and b), all three atypical pestiviruses formed a
272 monophyletic clade strongly supported by bootstrap value and posterior probability. The
273 position of this clade in the tree of E2 gene was same as that of Th/04_KhonKaen virus in
274 the tree based on full-length sequence analysis. As in the tree of Figure 1, this clade is
275 genetically distant from the established bovine pestivirus species BVDV-1 and BVDV-2,
276 as well as distinct from BDV, CSFV and pestivirus of Giraffe. The four recognized
277 species BVDV-1, BVDV-2, BDV and CSFV also formed monophyletic clades.

278

279 **4. Discussion**

280

281 Phylogenetic analysis is useful to classify novel viruses, and to reveal their evolutionary
282 history. Particularly for pestiviruses, whole-genome phylogeny has been used to classify
283 two novel species, Giraffe-1 and Reindeer-1 (Avalos-Ramirez et al., 2001), although the
284 latter has been re-classified as BDV (Becher et al., 2003).

285

286 In this study the complete genome sequence of the recently identified pestivirus
287 Th/04_KhonKaen was determined and analysed, aiming to establish the phylogenetic
288 positioning of this virus and to determine its relationship with known pestivirus species.

289 Following successful recovery of the virus from an inactivated serum sample, the genome
290 organization of Th/04_KhonKaen was shown to be reminiscent of that of an ncp
291 pestivirus, consistent with the recovery of a virus that did not induce CPE. The full-length

292 nucleotide identity of the Th/04_KhonKaen with the recognized BVDV species is around
293 68%, which is close to the range of 69.5% that separates BVDV-2 from BVDV-1 (Table
294 3), motivating its discrimination as a distinct pestivirus species of bovine origin.

295

296 A single phylogenetic tree topology was produced by all three methods, highly supported
297 by strong bootstrap and posterior probability values, on analysis of the full-length
298 sequence. This analysis unambiguously positioned the Th/04_KhonKaen virus as a unique
299 species in the consensus tree, distinct from the four established pestivirus species and
300 pestivirus of Giraffe, and closely related to the bovine species BVDV-1 and BVDV-2.

301 Remarkably, analysis of sequences of the complete N^{pro} and E2 coding region showed that
302 the three atypical pestiviruses formed a well-supported single clade, demonstrating that
303 these atypical bovine pestiviruses are genetically distinct from the current well established
304 pestivirus species. This relationship has been unequivocally confirmed by the analysis of a
305 sequence dataset combining the 5'UTR, and the complete N^{pro} and E2 gene regions of 56
306 pestiviruses, published in Liu et al. (2009).

307

308 Recovery of the Th/04_KhonKaen virus enables further studies currently in progress to
309 define cross-reactivity patterns with polyclonal and monoclonal antibodies, as well as the
310 characterization of in vitro and in vivo biological properties of this group of viruses.

311

312 In conclusion, the atypical pestivirus Th/04_KhonKaen was isolated from an inactivated
313 serum sample. Sequence analysis supported positioning of the Th/04_KhonKaen virus as a
314 new species within the pestivirus genus. Comparisons of sequences of the N^{pro} and E2

315 gene regions with those of the atypical pestiviruses D32/00_ 'HoBi', Brz buf 9, and CH-
316 KaHo/cont confirmed a high sequence similarity among these viruses, demonstrating that
317 they belong to a same, new group of pestiviruses of bovine origin.

318

319 **Acknowledgements**

320

321 The authors thank Dr. Karl Ståhl, The National Veterinary Institute, and Prof. Stefan
322 Alenius, Department of Clinical Sciences, Swedish University of Agricultural Sciences for
323 fruitful discussions. We are grateful to Dr. Hongyan Xia, Department of Biomedical
324 Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, for
325 valuable comments on the manuscript.

326

327 **References**

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

330 Becher, P., Avalos Ramirez, R., Orlich, M., Cedillo Rosales, S., Konig, M., Schweizer,
331 M., Stalder, H., Schirmer, H., Thiel, H.J., 2003. Genetic and antigenic characterization
332 of novel pestivirus genotypes: implications for classification. *Virology* 311, 96-104.

333 Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and
334 analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.* 41, 95-98.

335 Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic
336 trees. *Bioinformatics* 17, 754-755.

- 337 Liu, L., Xia, H., Wahlberg, N., Belák, S., Baule, C., 2009. Phylogeny, classification and
338 evolutionary insights into pestiviruses. *Virology* (doi:10.1016/j.virol.2008.12.004).
- 339 Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under
340 mixed models. *Bioinformatics* 19, 1572-1574.
- 341 Rumenapf, T., Thiel, H.-J., 2008. Molecular Biology of Pestiviruses, in: Mettenleiter T.C.,
342 Sobrino, F. (Eds), *Animal Viruses: Molecular Biology*. Caister Academic Press, Norfolk,
343 UK, pp.
- 344 Schirrmeier, H., Strebelow, G., Depner, K., Hoffmann, B., Beer, M., 2004. Genetic and
345 antigenic characterization of an atypical pestivirus isolate, a putative member of a novel
346 pestivirus species. *J. Gen. Virol.* 85, 3647-3652.
- 347 Ståhl, K., Kampa, J., Alenius, S., Persson Wadman, A., Baule, C., Aiumlamai, S., Belák,
348 S., 2007. Natural infection of cattle with an atypical 'HoBi'-like pestivirus—implications
349 for BVD control and for the safety of biological products. *Vet. Res.* 38, 517-523.
- 350 Stalder, H.P., Meier, P., Pfaffen, G., Wageck-Canal, C., Rüfenacht, J., Schaller, P.,
351 Bachofen, C., Marti, S., Vogt, H.R., Peterhans, E., 2005. Genetic heterogeneity of
352 pestiviruses of ruminants in Switzerland. *Prev. Vet. Med.* 72, 37-41.
- 353 Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary
354 Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24, 1596-1599.
- 355 Thiel, H.-J., Plagemann, P.G.W., Moenning, V., 1996. Pestiviruses, in: Fields, B.N.,
356 Knipe, D.M., Howley, P.M. (Eds.), *Fields Virology*, 3rd ed. Vol. 1, Lippincott–Raven,
357 Philadelphia, PA. pp. 1059–1073.
- 358 Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the
359 sensitivity of progressive multiple sequence alignment through sequence weighting,

- 360 position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673-
361 4680.
- 362 Xia, H., Liu, L., Wahlberg, N., Baule, C., Belák, S., 2007. Molecular phylogenetic
363 analysis of bovine viral diarrhoea virus: a Bayesian approach. *Virus Res.* 130, 53-62.

Accepted Manuscript

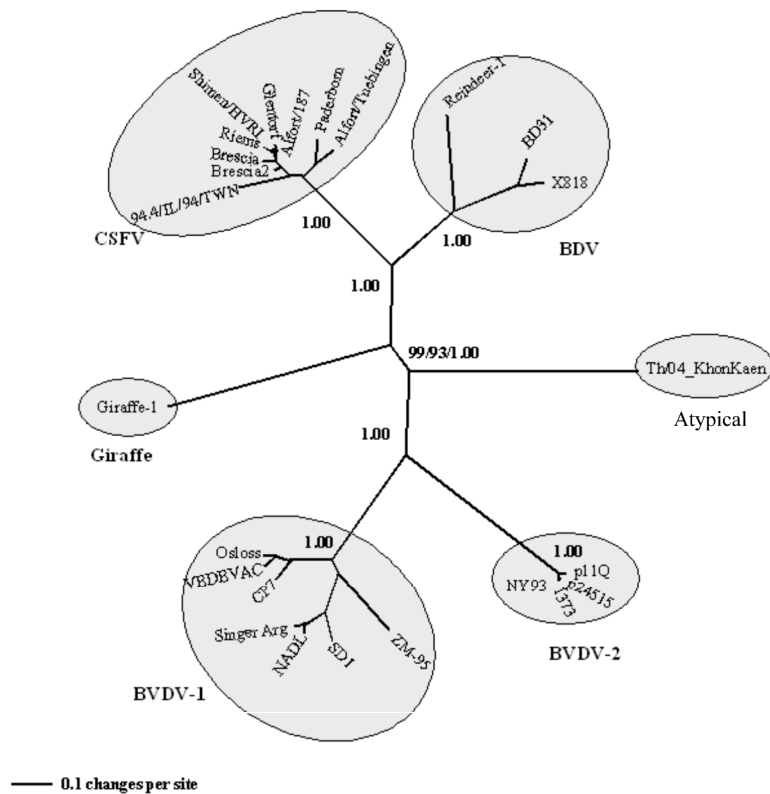


Fig. 1. Consensus phylogenetic tree based on analysis of the full-length sequence of the Th/04_KhonKaen virus and those of pestivirus species available in the GenBank. The Neighbor-joining tree was reconstructed under the nucleotide substitution model of Tamura 3-parameter using MEGA 4. PHYML (v2.4.4) was used for phylogeny inference according to maximum likelihood (ML) criterion, and MrBayes 3.1 for Bayesian inferring. The robustness of the tree was evaluated by bootstrapping 1000 replicates by Neighbor-joining and maximum likelihood, and by posterior probability from Bayesian analysis. The supporting value for major clades is shown in the order of NJ/ML/PP. "1.00" indicates 100%/100%/1.00. A representative, un-rooted consensus tree is shown in

the Figure. The bar indicates changes per site.

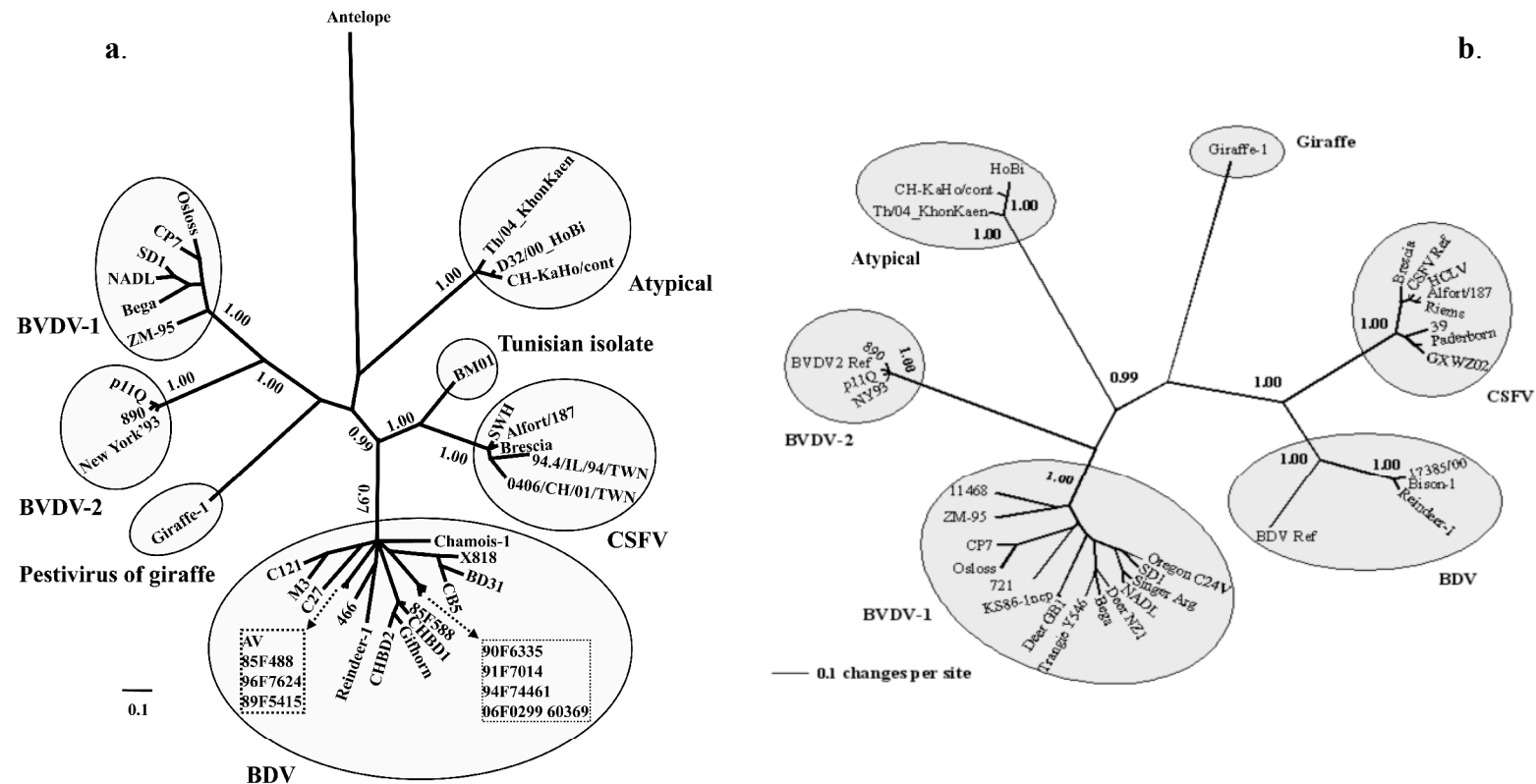


Fig. 2. Phylogenetic trees based on analysis of sequences of the complete N^{pro} (a., left) and E2 (b., right) gene regions. The relationship of atypical pestiviruses Th/04_KhonKaen, D32/00_'HoBi' and CH-KaHo/cont with representatives of established pestivirus species retrieved from GenBank is shown. The un-rooted consensus trees are inferred by Bayesian approach. Numbers indicate posterior probability values for major clades. The bar indicates changes per site.

Table 1. Nucleotide sequence of primers used for PCR amplification and sequencing of the Th/04_KhonKaen virus.

Primer name	Nucleotide sequence ^a	Position
TF1	GCTAGCCATGCCCTTAGTAGGA	98-119
TR1	CCCTAGTTCGGCCCATGAGTC	5382-5403
TF2	GGGCCGAAGTGGGTGGTGT	5390-5409
TR2	GTGCGTTGAGTGTAGTGTGTC	12247-12268
TF3	ATGGAGTTGTTAAACTTTGAAC	384-405
TR3	GCAGCTTCCTACCCAGATGG	868-878
TF5	GACCTCAGTTGTAAGCCTGAG	2457-2477
TR5	CCCCCTAGCTCCTTGTTTCAGTT	3554-3575
TF4	ACCCACCAAAAACAAACCAGA	1090-1111
TR4	TGGGCCTATCTCATTGGTCTCA	2495-2516
TF6	TCTTGGGTGGGAGGTATGTGCT	3505-3526
TR6	TTAGGCCCTTGCTTCTTTCTC	4648-4669
TF7	CAAGGCCCCAGAGCAAAGACG	4431-4451
TF8	CGAGCAGCGGCAGAATCAGTAT	5913-5934
TR8	GTCCGGCCAGTCCAACC	7120-7136
TF9	CAAATGCCATAGAATCAGGAGTA	6496-6518
TF10	TGATCACCCGGAACCCATACAG	6923-6944
TF11	AGGCAGGCTGGGGAGTGAAAC	7619-7639
TF12	AGGCAAAAGAATTAGCGGAAAGA	8317-8339
TR9	TGCCCAGGCT GAGT GCYTCYA	9649-9669
TR10	TCCTTCCCGTTCCAGTTGTTCT	10283-10304
TR11	GCGGCTTGTTACCTGAGTGTC	10965-10985
TF13	CTCTAAAATGAAGAAAGGGTGYG	10895-10917
TR12	CTGTGGTTTGCCAGATTCATAC	11327-11348
TR13	GGGGATATACATTTTGCCAGTC	11885-11906
TF14	AGGCACACCAGCAAGAGACTAC	11790-11811
PAS1	CTGTGTGCRTRARTGTAGTG	12252-12273
T3U-F1	TTATTACTTAGGCTGATGAGAAC	12214-12236
T5U-R1	CAGGGAGCTCACTGCCACCAC	139-159
GeneRacer RNA oligo	CGACUGGAGCACGAGGACACUGACA UGGACUGAAGGAGUAGAAA	
GeneRacer 5' primer	CGACTGGAGCACGAGGACACTGA	
RNA oligo 2	CGCAAUUGGGCGGUAGGCGUGCUGG CCGUCGUUUUAC	

^a Bold bases indicate mismatches with the sequence of the Th/04_KhonKaen virus.

Table 2. List of pestiviruses included in the analyses and their GenBank accession numbers

Species ^a	Pestivirus	Genomic sequence	N ^{pro}	E2
BDV	X818	NC_003679	g ^b	g
	BD31	U70263	- ^c	-
	Reindeer-1	NC_003677	g	g
	Bison-1	-	-	AF144619
	17385/00	-	-	AY163658
	Chamois_1	-	AY738083	-
	Gifhorn	-	AY163653	-
	CHBD1	-	AY895008	-
	CHBD2	-	AY895009	-
	466	-	AY163650	-
	CB5	-	AF145358	-
	M3	-	DQ273163	-
	C121	-	DQ273159	-
	C27	-	DQ273156	-
	AV	-	EF693962	-
	85F488	-	EF693963	-
	85F588	-	EF693966	-
	89F5415	-	EF693965	-
	90F6335	-	EF693968	-
	91F7014	-	EF693971	-
	94F74461	-	EF693974	-
	96F7624	-	EF693976	-
	06F0299 60369	-	EF696979	-
BVDV-1	ZM-95	AF526381	g	g
	SD1	M96751	g	g
	NADL	AJ133738	g	g
	Singer Arg	DQ088995	-	g
	CP7	U63479	g	g
	VEDEVAC	AJ585412	-	-
	Osloss	M96687	g	g
	Oregon C24V	-	-	AF091605
	Deer NZ1	-	-	AF144614
	Bega	-	AF049221	AF049221
	Trangie Y546	-	-	AF049222
	Deer GB1	-	-	AF144615
	KS86-1ncp	-	-	AB078950
	721	-	-	AF144609
	11468	-	-	AY734488
BVDV-2	p11Q	AY149215	g	g
	p24515	AY149216	-	-
	1373	AF145967	-	-
	NY93	AF502339	g	g
	890	-	g	U18059
CSFV	BVDV-2 Ref	-	-	NC_002032
	94.4/IL/94/TWN	AY646427	g	-
	Brescia	AF091661	g	g
	Brescia-2	M31768	-	-
	Riems	AY259122	-	g
	Shimen/HVRI	AY775178	-	-
	Glentorf	U45478	-	-
	Alfort/178	X87939	g	g
	Paderborn	AY072924	-	g
	Alfort/Tübingen	J04358	-	-
GXWZ02	-	-	AY367767	

^a Including unclassified pestiviruses

^b Same as genomic sequence

^c Sequence is not included in the analysis

	39	-	-	AF407339
	HCLV	-	-	AF091507
	CSFV Ref	-	-	NC_002657
	SWH	-	DQ127910	-
	0406/CH/01/TWN	-	AY568569	-
Tunisian isolates	BM01	-	AY453629	-
Pestivirus of giraffe	Giraffe-1	NC_003678	g	g
Atypical pestivirus	CH-KaHo/cont	-	AY895011	EU385605
	D32/00_HoBi	-	AY489117	AY604725
	Th/04_KhonKaen	FJ040215	g	g

Accepted Manuscript

Table 3. Identities of Th/04_KhonKaen with full-length sequences of 18 pestiviruses

	BD31	Reindeer -1	BDV /Ref	Oregon C24V	Osloss	SD1	ZM-95	BVDV-1 /Ref	1373	890	New York'93	p11Q	BVDV-2 /Ref	Alfort /187	Brescia	HCLV	Riems	CSFV /Ref	Giraffe-1
Th_04_KhonKaen	67.4	67.8	67.3	68.3	67.8	68.1	67.6	68.2	67.9	67.8	67.9	67.9	67.7	67.6	67.6	67.4	67.3	67.5	67.8
Giraffe_1	68.3	68.4	68.7	67.8	67.6	67.6	67.5	67.6	67.8	67.6	67.8	67.7	67.6	68	67.9	68	67.9	68.1	
CSFV/Ref	72	71.5	72.1	67.4	66.7	67.4	67.1	67.2	67	66.9	67	67.1	66.8	98	94.8	96.2	96.1		
Riems	71.5	71.1	71.6	67.5	66.7	67.5	66.9	67.3	67	67	67	67.1	66.8	96	93.2	99.2			
HCLV	71.6	71.2	71.6	67.5	66.7	67.5	66.9	67.3	67	66.9	67.1	67.1	66.8	96	93.2				
Brescia	71.7	71.7	72.1	67.5	67.1	67.2	67	67.2	66.7	66.6	66.7	66.7	66.5	94.9					
Alfort/187	71.9	71.5	72.1	67.5	66.8	67.4	67.1	67.4	66.9	66.9	66.9	66.9	66.8						
BVDV-2/Ref	66.9	67.3	66.9	69.4	69.6	69.6	69.6	69.5	96.4	96.3	96.3	96.4							
p11Q	67.1	67.4	67.2	69.6	69.7	69.9	70	69.7	96.8	96.3	96.7								
New York'93	67.2	67.4	67.2	69.6	69.8	69.9	70.1	69.8	99.4	96.1									
890	67.1	67.4	67	69.4	69.7	69.8	69.8	69.7	96.1										
1373	67.1	67.4	67.1	69.6	69.8	69.9	70	69.8											
BVDV-1/Ref	67.4	66.5	67.4	88.3	79.1	88.5	79.5												
ZM-95	67.2	67	67.5	79.9	78.7	79.8													
SD1	67.6	66.9	67.8	90.5	79.7														
Osloss	66.6	66.4	66.6	79.8															
Oregon C24V	67.3	66.8	67.3																
BDV/Ref	89.7	77																	
Reindeer-1	76.9																		