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Porcine Torque teno virus: Determination of viral genomic loads by genogroup-specific multiplex rt-PCR, detection of frequent multiple infections with genogroups 1 or 2, and establishment of viral full-length sequences

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1 **Porcine Torque teno virus: Determination of viral genomic loads by genogroup-specific**
2 **multiplex rt-PCR, detection of frequent multiple infections with genogroups 1 or 2, and**
3 **establishment of viral full-length sequences**

4

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17

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22

23 Genbank accession numbers of deposited sequences derived from this study:

24 Inserts of TTV1-S and TTV2-S rt-PCR standards; GQ466173 and GQ466174

25 TTV1#471819 and TTV2#472142 full-length sequences; GU188045 and GU188046

26 50 TTV1 and TTV2 UTR sequences; GU179292-GU179341

27 **Abstract**

28
29 *Torque teno virus* (TTV) is a non-enveloped virus with a circular, single-stranded DNA
30 genome. TTV is currently classified in the unassigned genus *Anellovirus*, and distinct TTVs
31 of tentative species-status infect a wide range of vertebrates. In domestic pigs and wild boars,
32 porcine TTV occurs in two genogroups, TTV1 and TTV2, which are currently detected using
33 only conventional PCR assays. To allow high-throughput testing, the present study describes
34 development of a multiplex real-time (rt)-PCR assay for efficient simultaneous detection of
35 TTV1 and TTV2. To demonstrate usefulness of this rt-PCR assay for large-scale testing, 203
36 serum samples from domestic pigs were screened for TTV infection. The detected rates of
37 single TTV1, single TTV2, and double TTV1/TTV2 infections were 32%, 17%, and 32% and
38 represent the first report on the occurrence of porcine TTV in Germany. In addition, 100 wild
39 boar lung samples were tested that confirmed high prevalences of TTV infection. Moreover,
40 establishment of genogroup-specific rt-PCR standards allowed the determination of mean
41 viral genomic loads in sera from TTV-infected swine to about $10^{4.5}$ /ml, respectively. To
42 verify the specificity of the rt-PCR assay, conventional PCR assays that amplify genogroup-
43 specific, size-distinguishable products from the TTV untranslated regions were designed. In
44 total, 50 clones derived from 24 PCR products obtained from 19 TTV1 and TTV2 single- or
45 double-infected animals were sequenced. Phylogenetic analyses of these sequences
46 demonstrated the frequent occurrence of multiple infections with distinct porcine TTVs of the
47 same genogroup. Moreover, two porcine TTV full-length sequences were established, one for
48 each genogroup.

49

50

51 **Keywords:**52 *Torque teno virus* (TTV); pig; real-time PCR; multiple infection; full-length sequence

53 1. Introduction

54

55 *Torque teno virus* (TTV) was discovered in samples from a human patient with
56 posttransfusion hepatitis of unknown etiology in 1997 (Nishizawa et al., 1997). TTV forms
57 small, icosahedral, non-enveloped virions with a diameter of about 32 nm (Itoh et al., 2000)
58 and has a circular, single-stranded genome of negative polarity that is, for human strains,
59 approximately 3.8 kb in length (Okamoto et al., 1998; Mushahwar et al., 1999; Miyata et al.,
60 1999). Together with the closely related TTV-like mini viruses that contain a smaller genome
61 of about 2.8-3 kb (Takahashi et al., 2000), TTVs demonstrate broad genetic variability and are
62 classified into the provisionally designated family *Circinoviridae* (Mushahwar et al., 1999),
63 tentative genus *Anellovirus* (Hino and Miyata, 2007). In addition to Anelloviruses exclusively
64 infecting humans, distinct TTVs have been detected in other vertebrate species including non-
65 human primates and tupaias (Leary et al., 1999; Verschoor et al., 1999; Okamoto et al., 2000),
66 farm animals such as cattle, sheep, and pigs (Leary et al., 1999), in cats, dogs (Okamoto et al.,
67 2002), and chickens (Leary et al., 1999), (Okamoto, 2009b).

68 The species-specific porcine TTVs are divided into two distinct genogroups, TTV1
69 (Okamoto et al., 2002) and TTV2 (Niel et al., 2005), that despite an overall similar genome
70 organization share only about 45% sequence identity. Porcine TTVs of both genogroups
71 infect domestic pigs (Okamoto et al., 2002; Niel et al., 2005) and wild boars (Martínez et al.,
72 2006; Kekarainen et al., 2007). Extensive studies investigating swine samples from Europe
73 (e.g. France (Bigarré et al., 2005), Italy (Martelli et al., 2006), Hungary (Takács et al., 2008),
74 and Spain (McKeown et al., 2004; Segalés et al., 2009)), Asia (China, Korea, Japan, and
75 Thailand (McKeown et al., 2004)), and North America (Canada (Brassard et al., 2008), USA
76 (McKeown et al., 2004)) demonstrate that these infections occur at variable, but consistently
77 high prevalences in piggeries worldwide. TTV infections of swine are therefore considered a

78 valuable model for the epidemiology of highly prevalent TTV infections in humans
79 (Kekarainen and Segalés, 2009).

80 A retrospective study demonstrated that porcine TTVs have been circulating in
81 European pig herds for more than 20 years (Segalés et al., 2009). Moreover, starting from an
82 extensive genomic variability, porcine TTV evolution during this period has progressed only
83 slowly, since even for the oldest samples investigated neither prevalence variation between
84 genogroups nor considerable phylogenetic clustering within genogroups were observed when
85 compared to TTV sequence information obtained from more recently collected sera (Segalés
86 et al., 2009). The routes of infection with porcine TTV are highly diverse, and both horizontal
87 and vertical transmission routes have been identified that contribute to TTV spread among
88 pigs to probably distinct, but complementary extents. Currently, orofecal transmission is
89 considered the major route of horizontal infection since porcine TTVs have been detected in
90 feces (Brassard et al., 2008), but virus-containing secretions, e.g. saliva, may be as important.
91 Also, congenital transmission may be possible, since TTV has been detected in boar semen
92 (Kekarainen et al., 2007). In addition, recent studies demonstrate that intrauterine infections
93 (Pozzuto et al., 2009; Martínez-Guinó et al., 2009) and virus-containing colostrum (Martínez-
94 Guinó et al., 2009) represent relevant pre- and postpartum routes of vertical infection. These
95 multiple routes of transmission account for the high prevalence of both genogroups
96 worldwide and support the belief that porcine TTV is highly contagious.

97 Nevertheless, it has not yet been established whether infection with porcine TTV
98 causes a specific disease. Although it has been found that TTV1 alone does not induce a
99 specific disease (Krakowka and Ellis, 2008), it appears that TTV1 might contribute to severer
100 disease syndromes during coinfections with other porcine viruses (Krakowka et al., 2008;
101 Ellis et al., 2008) and that TTV2 might be associated with miscarriage in sows (Sibila et al.,
102 2009). Importantly, as has been described for porcine circovirus 2 (Liu et al., 2000) and
103 suggested for human TTV (Okamoto, 2009a), the relative prevalence of particular TTV

104 genogroups and/or the amounts of virus in the host might affect likelihood of causing or
105 exacerbating specific diseases or syndromes.

106 Extensive prevalence studies involving large numbers of samples are needed to
107 elucidate the pathogenic potential of porcine TTVs. However, the TTV diagnostic capability
108 is still limited. Currently, there is no cell culture system or serological assay available, and
109 TTV diagnosis is limited to conventional PCR, including non-nested (Segalés et al., 2009),
110 nested (Kekarainen et al., 2006), or rolling-circle amplification (Niel et al., 2005) assays.
111 There is currently no method for high throughput testing of swine samples. In the present
112 study, a sensitive multiplex TTV genogroup-specific real-time (rt)-PCR assay was
113 established. Demonstrating its feasibility for epidemiological surveillance, this rt-PCR assay
114 was used to determine the prevalences of both genogroups in two hundred and three swine
115 serum samples from three German pig herds and in one hundred lung samples from wild
116 boars. Moreover, this assay allowed to quantify the virus genomic loads in TTV-infected pigs.
117 To confirm specificity of the established rt-PCR, a phylogenetic analysis of some of the TTV
118 strains detected was performed that revealed the frequent occurrence of multiple infections
119 with distinct porcine TTV strains of the same genogroup. Finally, two full-length porcine
120 TTV sequences were established, one for each genogroup. Procedures and implications of this
121 work are described and discussed below.

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130 2. Materials and methods

131

132 2.1. Blood sampling and DNA extraction

133

134 Two hundred and three pigs from three independent herds were sampled. From each
135 animal, 10 ml venous blood was taken from the *vena jugularis* or the *vena cava cranialis* and
136 stored at 4°C in 10 ml Kabevette syringes (Kabe, Nümbrecht-Elsenroth, Germany). For
137 separation of serum, clotted blood was centrifuged for 5 min at 3,360 g. DNA from 200 µl of
138 each serum sample was extracted using a blood kit according to the manufacturer's
139 instructions (QIAamp 96-well DNA blood kit, Qiagen, Hilden, Germany), eluted in a volume
140 of 140 µl AE-buffer, and stored at -20°C until used for PCR. For DNA extraction from wild
141 boar lungs, organ samples were homogenized in PBS, centrifuged for 1 min at 2,500 g, and
142 200 µl of the resulting supernatant were used for DNA extraction using the QIAamp 96-well
143 DNA blood kit.

144

145 2.2. Primers and rt-PCR probes

146

147 Sequences of sense primers and binding sites of anti-sense primers (Eurofins MWG,
148 Ebersberg, Germany) used for conventional PCRs and rt-PCRs are given in Fig. 1. Probes for
149 rt-PCR (Applied Biosystems Deutschland GmbH, Darmstadt, Germany) bear a 3'-terminal
150 MGB quencher. Sequences of probes are given in Fig. 1. *TTV1 probe* is 5'-FAM-labelled,
151 while *TTV2 probe* bears a 5'-VIC-label. Primers and probes used for PCR were designed
152 using the Primer Express 3.0 software (Applied Biosystems Deutschland GmbH).

153

154

155

156 2.3. Conventional PCR

157

158 Conventional PCRs for the amplification of TTV1 or TTV2 UTR sequences were
159 performed using pan-TTV anti-sense oligonucleotide *panTTV as* and sense primers *TTV1 s* or
160 *TTV2 s*, respectively (Fig. 1). In a total volume of 50 μ l per sample, 0.4 μ l of Taq polymerase
161 (Qiagen) was mixed with 5 μ l 10xQiagen buffer, 5 μ l Q-solution, 2 μ l of 25 mM MgCl₂, 1.5
162 μ l 20 mM dNTPs, 29.1 μ l distilled water, 1 μ l of each 20 mM primer, and 5 μ l of sample
163 DNA in AE-buffer (see above). Cycling then was performed as described elsewhere (Segalés
164 et al., 2009). For detection of TTV-specific PCR products, electrophoresis was performed
165 with 5 μ l of each reaction loaded on a 1% agarose gel containing ethidium bromide that was
166 subsequently transilluminated on a 254 nm UV-screen and photo-documented (Fig. 2).

167

168 2.4. Cloning of PCR products, transformation of bacteria, and plasmid preparation

169

170 Forty-five μ l of conventional PCR product were loaded on a 1% agarose gel,
171 electrophoresized, and bands of about 300 bp were excised, purified (QIAquick gel extraction
172 kit, Qiagen) and eluted in 30 μ l of distilled water. After assessing the DNA concentrations of
173 purified PCR products by agarose gel electrophoresis and UV-transillumination,
174 approximately 25 ng of insert were ligated into 50 ng of vector pDrive (PCR cloning kit,
175 Qiagen) at 14°C over night, respectively. The ligation product then was transformed into
176 TOP10 *Escherichia coli* bacteria by heat-shocking, and the reaction was plated onto LB
177 medium/agarose plates containing ampicillin, X-Gal, and IPTG following standard
178 procedures recommended by the cloning kit manual. After incubation at 37°C for 16 hrs,
179 white colonies were used to inoculate 4 ml of LB-medium containing ampicillin and
180 incubated for another 16 hrs at 37°C in a shaking incubator. Recombinant plasmids then were
181 extracted (Wizard Plus SV Miniprep kit. Promega, Madison, WI, USA) and preparations

182 containing plasmids with the correct inserts were identified by digestion with restriction
183 enzymes *NotI* and *MluI* (New England Biolabs Deutschland GmbH, Schwalbach, Germany),
184 subsequent agarose gel electrophoresis, and UV-transillumination of resulting DNA
185 fragments.

186

187 *2.5. Nucleotide sequencing, assembling of sequences, and phylogenetic analysis*

188

189 Nucleotide sequencing was performed using the Thermo Sequenase DYEnamic Direct
190 Sequencing Kit (GE Healthcare, Buckinghamshire, UK), about 1 µg of plasmid DNA, 5'-
191 IRD-700- or 5'-IRD-800-labelled standard sequencing primers (Eurofins MWG), and a LI-
192 COR IR² DNA sequencer (LI-COR Inc., Lincoln, NE, USA). The obtained nucleotide
193 sequences of PCR inserts then were checked for fidelity and assembled using the e-Seq V3.0
194 and AlignIR V1.1 software packages (LI-COR Inc.).

195 TTV sequences were aligned using the CLC Main Workbench 4.1.1 software package (CLC
196 bio, Aarhus, Denmark) at gap open cost of 10, gap extension cost of 1, end gap cost setting
197 free, under slow (very accurate) settings. If indicated, alignments were used as input files to
198 perform a distance-based phylogenetic analysis by the neighbour joining algorithm followed
199 by bootstrap analysis in 1,000 replicates or to determine relative sequence identities (CLC
200 Main Workbench 4.1.1 software package, CLC bio).

201

202 *2.6 Generation of rt-PCR standards for both TTV genogroups*

203

204 TTV1-specific rt-PCR standard TTV1-S contains a TTV1-specific PCR product of
205 317 bp (GenBank accession number GQ466174) that was amplified from serum 3 using
206 primers *panTTV as* and *TTV1 s* and ligated into 3851 bp vector pDrive (PCR cloning kit,
207 Qiagen). For generation of TTV2-specific rt-PCR standard TTV2-S, a 309 bp fragment

208 amplified from serum 19 (GenBank accession number GQ466173) using primers *TTV2 s* and
209 *rt-PCR TTV2 as* (Fig. 1) was cloned into plasmid pDrive. Presence of genogroup-specific rt-
210 PCR primer binding sites was confirmed by nucleotide sequencing, respectively. When used
211 for rt-PCRs, standards were serially diluted in distilled water or TE buffer.

212

213 *2.7. Multiplex real-time (rt)-PCR*

214

215 The rt-PCR assays were performed in 384-well plates using an ABI 7900HT Fast
216 Real-Time PCR System (Applied Biosystems Deutschland GmbH). The multiplex rt-PCR
217 master mix of 13 μ l per replicate contained 8 μ l Taqman 2xUniversal PCR Master Mix
218 (Applied Biosystems Deutschland GmbH), 0.2 μ l of distilled water, 1.5 μ l of both 2.5 μ M
219 probes, and 0.45 μ l of each of the four TTV-specific 20 mM primers. 3 μ l of sample DNA in
220 AE-buffer (see above) per replicate then were added to the Master Mix, and rt-PCRs were
221 performed in two or three replicates starting with an initial inoculation at 50°C for 2 min,
222 followed by a denaturation step at 95°C for 10 min. Cycling then was performed in forty-five
223 cycles at 95°C for 15 sec followed by 60°C for 1 min. When fully loaded, a 384-well plate
224 included two negative template controls where distilled water was used as rt-PCR template
225 and four negative elution controls where DNA preparation eluate from distilled water served
226 as template for rt-PCR. To calculate TTV genomic loads per ml of investigated sera,
227 individual results from quantitative rt-PCRs were multiplied by 233.33 (140 μ l DNA
228 preparation eluate from 200 μ l serum \times 5 / 3 μ l PCR input).

229

230 *2.8 Statistical analysis of rt-PCRs*

231 For inter- and intra-assay evaluation of rt-PCRs, software STATISTICA (StatSoft,
232 Tulsa, OK, USA) was used. Coefficient of variance given as percentage is defined as the
233 ratio of the standard deviation to the mean value.

234 **3. Results**

235

236 *3.1 Establishment of a multiplex rt-PCR for the simultaneous detection of both porcine TTVs*

237

238 The TTV untranslated region (UTR) is usually targeted for differentiating between
239 TTV genogroup 1 (TTV1) and TTV genogroup 2 (TTV2) using genogroup-specific
240 conventional PCR assays and sequencing of the amplicons (Kekarainen et al., 2007; Segalés
241 et al., 2009). Therefore, to establish genogroup-specific real-time (rt)-PCRs, sequences of
242 published full-length and subgenomic porcine TTV sequences were aligned in their viral
243 UTRs to identify conserved motifs for the design of rt-PCR primers and probes (Fig.1 and
244 data not shown). Using these alignments, two non-overlapping sets of primer/probe
245 combinations were designed that each were expected to be genogroup-specific and that
246 correspond to or bind at nucleotides 11-66 of TTV1 reference strains 1p and TTV1 Sd-TTV31
247 (primers *rt-PCR TTV1 s* and *rt-PCR TTV1 as*, probe *TTV1 probe*) or nucleotides 246-309 of
248 TTV2 reference strain 2p (primers *rt-PCR TTV2 s* and *rt-PCR TTV2 as*, probe *TTV2 probe*);
249 (Fig. 1). The two established sets of TTV1- and TTV2-specific primers and probes then were
250 evaluated for their applicability in a multiplex rt-PCR assay to simultaneously detect
251 sequences from both porcine TTV genogroups.

252 To confirm high efficacy and sensitivity and to allow evaluation of the established
253 TTV genogroup-specific rt-PCR protocol (see Materials and methods), plasmids TTV1-S and
254 TTV2-S were generated as rt-PCR standards specific for TTV1 and TTV2 (see Materials and
255 methods). Both standards were investigated by multiplex rt-PCR in a calculated plasmid input
256 range from 10^8 to 10^0 rt-PCR standard molecules per sample, respectively. For evaluation of
257 intra- and inter-assay variation, three 384-well plates were analyzed in separate runs that each
258 contained three independent standard curves of both rt-PCR standards in three replicates. The
259 obtained results demonstrated the efficient detection of both standards over a range of eight

260 decalarithmic scales. Detection of 10^1 genome equivalents occurred around cycle 34 for
261 TTV1-S and cycle 33 for TTV2-S for all replicates, while 1 genome equivalent was not
262 consistently detected for both standards (data not shown). Based on ct-values, in all three
263 assays the intra-assay variations of detecting TTV standards in a range from 10 to 10^4 genome
264 equivalents were below a coefficient of variance (CV) of 1.7% for TTV1-S and below 12.7%
265 for TTV2-S. The respective inter-assay variations were below a CV of 2.1% for TTV1-S and
266 below 10.3% for TTV2-S (data not shown). Determined average slopes and R² values of
267 standard curves were -3.41 and 0.997 for TTV1-S and -3.52 and 0.997 for TTV2-S detection.
268 Accordingly, efficacies of TTV1 and TTV2 rt-PCR amplification are largely optimized. In
269 view of the fact that the developed primer/probe sets each target highly conserved porcine
270 TTV sequences, a cut-off cycle threshold of 38 cycles was set for evaluation of results, thus
271 excluding false positives typically caused by the amplification of non-specific sequences in
272 very late rt-PCR cycles. To assess intrinsic specificity of the established assay, we performed
273 multiplex rt-PCR runs investigating sets of serially diluted PCR standards TTV1-S or TTV2-S
274 in which one of the primers, probes, standards, or the enzyme master mix were replaced by an
275 equal volume of distilled water, respectively. Our results demonstrate that each of the
276 genogroup-specific primers, the probe, and the master mix were essential for efficient
277 detection of one TTV genogroup, respectively, while presence of primers and the probe
278 designed for detection of the other genogroup were not (data not shown). Moreover, only
279 TTV1-specific but no TTV2-specific amplicons were detected when TTV1-S was used and
280 only TTV2-specific but no TTV1-specific amplicons were detected when TTV2-S was used,
281 thus demonstrating that no cross-detection between TTV1 and TTV2 occurred. Taken
282 together, this multiplex rt-PCR assay allows the efficient, specific, reproducible, and highly
283 sensitive detection of both porcine TTV genogroups down to 10 genome equivalents or less in
284 a 3 μ l sample preparation input volume. This corresponds to 2,333 genome equivalents in one
285 ml of serum.

286 3.2. Prevalences of TTV infection and quantification of viral loads

287

288 Using the established multiplex rt-PCR, DNA preparations from 203 serum samples of
289 domestic pigs collected from three independent German piggeries were analyzed for porcine
290 TTV1 and TTV2. Results of the multiplex assay demonstrated that 20% of the investigated
291 samples did not contain detectable sequences of porcine TTV1 or TTV2, while 32% and 17%
292 were positive for TTV1 or TTV2 only, respectively, and 32% were positive for both (Table
293 1). Moreover, 100 DNA preparations from wild boar lung samples were analyzed using the
294 established multiplex rt-PCR. Here, prevalences for single TTV1 or single TTV2 detection
295 were 20% and 49%, respectively, while 19 % were positive for both TTVs and 20% were
296 tested negative (Table 2). To exclude intrinsic rt-PCR inhibition by the material tested, all
297 samples negative for TTV were investigated by rt-PCR using the VIC-based Taqman
298 Exogenous Internal Positive Control (Applied Biosystems). Here, all samples showed ct-
299 values similar to the positive control of the test-kit. Thus, samples tested negative for TTV did
300 not contain intrinsic rt-PCR inhibitors. In addition, all samples analyzed for TTV were also
301 tested for *Porcine Circovirus 2* (PCV2) and *Porcine Respiratory Syndrome Virus* (PRRSV)
302 EU and US strains by rt-PCR (data not shown). Here, detection or no detection of TTV
303 occurred independently from whether samples were positive or negative for PCV2 or PRRSV
304 (data not shown), demonstrating that specificity of the TTV rt-PCT was not influenced by the
305 presence or absence of other viral swine pathogens.

306 Finally, to determine absolute viral genomic loads in TTV-infected swine by use of the
307 established rt-PCR standards, a set of 133 samples from sera of domestic pigs was analyzed
308 by TTV-specific multiplex rt-PCR. For the twenty-six samples tested positive for TTV1 only,
309 the forty-four tested positive for TTV2 only, and the forty-four samples tested positive for
310 both TTVs the determined average viral genomic loads were about $10^{4.5}$ /ml of prepared

311 serum, respectively (Table 3). Interestingly, the determined genomic TT-viral loads between
312 individual animals varied in a range of 1,000-fold and more for both genogroups (Table 3).

313

314 *3.3 Genogroup-specific conventional PCRs confirm results from rt-PCR*

315

316 Both genogroup-specific rt-PCR primer/probe sets used for multiplex assays yielded
317 prevalence rates similar to those reported from conventional PCR analyses of swine sera in
318 other studies, e.g. (Martínez et al., 2006; Sibila et al., 2009). To confirm specificity of the rt-
319 PCR results, two genogroup-specific pairs of oligonucleotides consisting of sense primers
320 *TTV1 s* or *TTV2 s* (Segalés et al., 2009) and, based on a viral UTR sequence alignment, one
321 common conserved anti-sense primer, *panTTV as*, were designed (see Fig. 1 and Materials
322 and methods). These genogroup-specific pairs of primers were expected to amplify genomic
323 fragments of about 320 nucleotides (nts) for TTV1 or 270 nts for TTV2 (Fig. 1). Using these
324 sets of primers, conventional PCRs were performed analyzing DNA preparations from serum
325 samples that were previously tested by TTV-specific multiplex rt-PCR. In total, 20 samples
326 were randomly selected comprising seven sera that were tested positive only for TTV1 (sera
327 1-7), six that were tested positive only for TTV2 (sera 14-19), and six that were tested
328 positive for both (sera 8-13), while water and a sample tested negative for TTV1 and TTV2
329 served as negative controls. Conventional PCR products then were loaded onto an agarose
330 gel, and gel electrophoresis was performed together with flanking molecular markers (Fig. 2).
331 The results demonstrated amplification of DNA fragments slightly larger than 300 basepairs
332 by the TTV1-specific conventional PCR for all 13 sera previously tested positive for TTV1,
333 while analysis of 11 of 12 sera previously tested positive for TTV2 generated uniform DNA
334 products slightly smaller than 300 bp in the TTV2-specific conventional PCR. Negative
335 controls did not lead to amplification of described products in either conventional PCR (Fig.
336 2). When assessing sensitivity of both conventional PCRs, serial dilutions of rt-PCR standards

337 TTV1-S and TTV2-S were only detected down to 10^2 input molecules (data not shown).
338 Thus, sensitivity of the established multiplex rt-PCR is at least 10-fold higher than that of the
339 conventional PCRs. Taken together, the established conventional PCRs confirm the results
340 obtained from porcine TTV-specific multiplex rt-PCR by amplifying products of expected
341 and genogroup-specific sizes from a sample fraction of adequate coverage.

342

343 *3.4 Phylogenetic analysis of porcine TTV UTR sequences*

344

345 To verify TTV- and genogroup-specificity of amplicons from conventional PCRs (see
346 above) by nucleotide sequencing, all 13 TTV1-specific products and the 11 TTV2-specific
347 products depicted in Fig. 2 were ligated into a bacterial cloning vector and transformed into
348 bacteria. Following cultivation of selected single bacteria colonies and subsequent plasmid
349 preparation, 2-3 DNA samples per cloned PCR product each containing the correct insert
350 were subjected to bidirectional nucleotide sequencing. The obtained data then was checked for
351 fidelity and assembled, and identity of the obtained 50 sequences from 24 cloned PCR
352 products was checked by the Blastn software package (NCBI, Bethesda, USA). According to
353 Blastn results, all sequences analyzed clearly were of porcine TTV origin (data not shown).
354 Subsequently, the 50 sequences (Genbank accession numbers GU179292-GU179341) were
355 aligned with each other and to TTV UTR sequences from porcine reference strains of both
356 genogroups (1p and TTV Sd-TTV31 for TTV1, 2p for TTV2; (Okamoto et al., 2002; Niel et
357 al., 2005)) as well as to canine TTV outgroup strain dog Cf-TTV10-1 (Okamoto et al., 2002).
358 The alignment then was used to perform a distance-based phylogenetic analysis by the
359 neighbour joining algorithm. To assess the confidence limits of the branching pattern, a
360 bootstrap analysis in 1000 replicates was included. With high confidence, the resulting tree
361 showed clustering of two distinct TTV genogroups that differed from outgroup strain dog Cf-
362 TTV10-1 (Fig 3 A). Within these clusters, as was confirmed by Blastn results (data not

363 shown), all sequences belonged either to porcine TTV1 or TTV2, respectively (Fig. 3 A).
364 When performing a maximum likelihood phylogeny analysis instead of using the neighbour
365 joining algorithm, a subsequent bootstrap analysis in 1000 replicates resulted in a very similar
366 tree and clusters, branching patterns, and bootstrap values (data not shown). As indicated by
367 names and numbering in Fig. 3 A, all sequences in the TTV1 and TTV2 clusters originated
368 from conventional genogroup-specific PCR analysis of samples tested positive for the
369 respective genogroup by multiplex rt-PCR (Fig. 2). Thus, specificity of the genogroup-
370 specific conventional PCRs and, accordingly, that of the established genogroup-specific rt-
371 PCRs was irrefutably confirmed by sequencing and phylogenetic analysis.

372 Interestingly, when homologies between sequences obtained from the same
373 conventional PCR products, and thus, from the same animals, were investigated it was found
374 that values were not always 99% or higher, but frequently and considerably lower,
375 demonstrating sequence identities down to 92.4% for TTV1 (TTV1-13-1 and TTV1-13-2) and
376 down to 85.1% for TTV2 (TTV2-13-1 and TTV2-13-2); Fig. 4 and data not shown. Also,
377 within the relatively small sample number investigated, the extent of heterogeneity of TTV
378 sequences identified from individuals was not evenly prominent for both porcine TTV
379 genogroups, but more noticeable for TTV2. Considerably distinct TTV2 sequences were
380 found in 7 of 11 sera, respectively, whereas this was found in only 4 of 13 sera for TTV1 (Fig.
381 3 B, brackets to the right).

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383 *3.5 Establishment of porcine TTV full-length sequences*

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385 Currently, there is only one complete porcine TTV full-length sequence published, TTV1 Sd-
386 TTV31 (Okamoto et al., 2002). In addition, two almost complete full-length sequences exist,
387 TTV1 1p and TTV2 2p (Niel et al., 2005), that lack sequence information in the viral GC-rich
388 region. To establish additional complete porcine TTV full-length sequences, DNA

389 preparations from two porcine sera were used that were previously tested positive only for
390 TTV1 (serum 3) or only for TTV2 (serum 19) both by multiplex and conventional PCRs (Fig.
391 2). Based on published sequences and the viral UTR sequence information initially obtained
392 from generating rt-PCR standards TTV1-S and TTV2-S (see Materials and methods),
393 extensive TTV-specific PCR analyses were performed (data not shown). Eventually, a total of
394 six (serum 3) and eleven (serum 19) PCR fragments was obtained that were purified and
395 cloned into a bacterial vector, respectively (data not shown). Bidirectional nucleotide
396 sequencing of at least three independent cDNA clones for each PCR product followed by
397 sequence analyses confirmed their porcine TTV origin, respectively (data not shown). Finally,
398 the overlapping consensus sequences of the cloned and sequenced PCR products were
399 assembled to two circular and complete porcine TTV full-length sequences, TTV1#471819
400 for sequences amplified from serum 3 and TTV2#472142 for PCR products obtained from
401 serum 19.

402 TTV2#472142 represents a complete porcine TTV2 genomic sequence with a size of
403 2,802 nucleotides (Genbank accession number GU188045), while TTV1#471819 is a
404 complete porcine TTV1 genomic sequence with a size of 2,863 nucleotides (Genbank
405 accession number GU188046). As observed previously when comparing porcine TTVs (Niel
406 et al., 2005), the sequences of Sd-TTV31, 1p, 2p, TTV1#471819, and TTV2#472142 shared a
407 remarkable similarity in their overall genomic organization, including the conserved presence
408 and locations of a putative TATA-box, a viral UTR, ORF1, ORF2, ORF3 resulting from
409 mRNA-splicing, and a GC-rich region (Fig. 5 A), sequence elements that were initially
410 described by Okamoto et al., 2002.

411 To assess the genetic relatedness of the determined TTV full-length sequences to each
412 other and to the previously published sequences, pairwise sequence alignments were
413 performed to obtain relative identities on nucleotide level. Here, TTV1#471819 shared 85%
414 sequence homology with 1p and 68% with Sd-TTV31, but only 45% with TTV2#472142 and

415 48% with 2p. Conversely, TTV2#472142 shared 88% homology with 2p, but only 43-45%
416 with TTV1#471819, 1p, and Sd-TTV31 (Table 4). Accordingly, when a distance-based
417 phylogenetic analysis was performed, TTV2#472142 and 2p clustered into one group, while
418 TTV1#471819, 1p, and Sd-TTV31 clustered into a different group in which TTV1#471819
419 and 1p were more closely related to each other than to Sd-TTV31, respectively (Fig. 5 B).

420 Similarly, alignments between the deduced hypothetical viral proteins were performed
421 to obtain relative sequence identities on amino-acid level. TTV1#471819 ORF1 protein
422 shared 85% identity with that of 1p and 49% with that of Sd-TTV31, but only 27% with those
423 from 2p or TTV2#472142. TTV2#472142 ORF1 protein shared 85% identity with that of 2p,
424 but only 28% or less with those from the other TTVs. TTV1#471819 ORF2 protein shared
425 96% identity with that of 1p and 50% with that of Sd-TTV31, but only 29% or less with those
426 from 2p or TTV2#472142. TTV2#472142 ORF2 protein shared 90% identity with that of 2p,
427 but only 32% or less with those from the other TTVs. Finally, TTV1#471819 ORF3 protein
428 shared 85% identity with that of 1p and 46% with that of Sd-TTV31, but only 19% or less
429 with those from 2p or TTV2#472142.

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441 **4. Discussion**

442 Until now, there was no diagnostic method available to investigate the prevalences of
443 porcine TTV infections in high-throughput assays or to quantify genogroup-specific viral
444 genomic loads. In this study, two non-overlapping primer/probe combinations for the
445 detection of both TTV genogroups were designed to establish the first porcine TTV-specific
446 multiplex rt-PCR assay described. This assay allows the convenient, specific, efficient, and
447 highly sensitive simultaneous detection of both TTV genogroups. Using this multiplex rt-
448 PCR, hundreds of swine serum samples from several piggeries and from wild boars were
449 investigated, thus demonstrating applicability of this test for large scale epidemiological
450 surveillance of TTV and providing an estimation of the prevalences of infection with porcine
451 TTV genogroups in Germany. Moreover, by use of newly designed genogroup-specific rt-
452 PCR standards, absolute genogroup-specific viral genomic loads in sera from TTV-infected
453 swine were determined. Also, by sequence analyses the apparently frequent occurrence of
454 multiple infections with distinct TTVs of the same genogroup was discovered. Finally, this
455 study provides two new porcine TTV full-length sequences, TTV1#471819 and
456 TTV2#472142.

457 Sequence analyses of the latter that included previously published porcine TTV
458 sequences confirmed earlier observations on the conserved overall genome organization
459 shared by porcine TTV1 and TTV2 (Okamoto et al., 2002; Niel et al., 2005). However, our
460 analyses also demonstrated that TTV1 and TTV2 share only remarkably low sequence
461 identities, both on nucleotide and on amino-acid levels (see above). It therefore appears to the
462 authors that TTV1 and TTV2 which share only up to 48% nucleotide sequence identity and
463 only 32% or less amino-acid identities of the deduced viral proteins should not further be
464 considered as genogroups of the same virus species, but as distinct species. This conclusion is
465 shared and supported by other researchers with similar and very recently published findings
466 (Huang et al., 2009).

467 The role of TTV infections in swine and its association with disease induction,
468 however, is still unclear. Recent studies suggest that TTV1 alone causes only moderate
469 histologic changes and no disease in gnotobiotic pigs (Krakowka and Ellis, 2008), but favors
470 the appearance of severe disease syndromes in coinfections with other porcine viruses such as
471 the *Postweaning Multisystemic Wasting Syndrome* (PMWS) during infections with PCV2
472 (Ellis et al., 2008) or the *Porcine Dermatitis and Nephropathy Syndrome* (PDNS) during
473 infections with PRRSV (Krakowka et al., 2008). Similarly, TTV2 might be involved in the
474 occurrence of PMWS (Kekarainen et al., 2006) or statistically associated with miscarriage in
475 sows (Sibila et al., 2009).

476 Therefore, to further elucidate the interdependence of TTV infection and the induction
477 of disease, adequate diagnostics are needed to allow high-throughput analyses of a
478 statistically meaningful significance (Okamoto, 2009a). As already implemented for human
479 TTV (Kato et al., 2000), this study now provides rt-PCR tools for directed research on porcine
480 TTV. The established assay not only allowed the convenient and reliable investigation of high
481 sample numbers, but also demonstrated a sensitivity superior to that of unnested conventional
482 PCRs (see Results), probably due to the inherent amplification of shorter sequence stretches
483 and a higher sensitivity of the rt-PCR cycler's read-out hardware (e.g., Fig. 2 B, lane 11 and
484 data not shown). The rt-PCR was used to assess TTV1 and TTV2 prevalences, and the
485 obtained values of these initial investigations confirm that infections with porcine TTVs are
486 highly prevalent in Germany both in domestic pigs and in wild boars. Exclusive TTV1
487 infections and TTV1/TTV2 double-infections occurred more frequently in domestic pigs,
488 while exclusive TTV2-infections were more frequently detected in wild boars. These
489 observations are consistent with similar investigations based on conventional PCRs, where
490 variable but consistently high prevalences of TTV infections in swine were detected
491 (Kekarainen et al., 2006; Kekarainen et al., 2007; Segalés et al., 2009). Further studies
492 investigating representative sample numbers from selected swine subpopulations are needed

493 to further characterize these initial findings and studies by others on the role of age (Martínez
494 et al., 2006; Sibila et al., 2009), gender (Martínez et al., 2006), breed, origin from distinct
495 herds, or geographical regions (compare (Martínez et al., 2006; Kekarainen et al., 2007;
496 Segalés et al., 2009), and different types of pig housing or penning (Martelli et al., 2006) on
497 porcine TTV infection and genogroup prevalences. Moreover, additional focus on diseased
498 animals will contribute to a greater understanding of porcine TTV as a potential pathogen.

499 Importantly, by use of the established quantitative rt-PCR assay it now is possible to
500 determine the relative and absolute porcine genogroup-specific TT viral genomic loads (Table
501 3). As determined in our initial investigations, the average absolute viral genomic loads were
502 about $10^{4.5}$ per ml of porcine serum for TTV1 and TTV2. Interestingly, for both TTVs the
503 determined genomic loads showed a remarkably high variation of 1,000-fold and more
504 between individual animals (Table 3). Investigating this variation of TT viral genomic loads
505 in single- and double-infected animals and correlating these values to clinical data will be of
506 utmost importance for future investigations. It was only such rt-PCR-based studies that
507 elucidated the importance of the viral genomic load in disease induction and contributed to
508 the identification of PCV2 as an important swine pathogen (Olvera et al., 2004). In addition,
509 the established rt-PCR can be used to determine organ-specific viral genomic loads to further
510 investigate observed genogroup-specific organ tropisms of porcine TTV (Bigarré et al., 2005).

511 As was described in other studies (Kekarainen et al., 2006; Martínez et al., 2006;
512 Segalés et al., 2009), this study demonstrates that all obtained TTV sequences clearly cluster
513 either into genogroups 1 or 2 (Fig. 3 A). Also, the data confirm reports that double-infections
514 with TTV strains from both genogroups occur frequently (Kekarainen et al., 2006; Martínez
515 et al., 2006); (Tables 1 and 2). Moreover, when evaluating the data for individual animals, in
516 some cases sequence variations in a range of about 1% between the molecular clones
517 originating from the same PCR product and thus, from the same animal were identified (Fig.
518 3 B, small brackets to the right, e.g., TTV1-9-1 and TTV1-9-2, and data not shown). This can

519 be explained by the described emergence of TTV quasispecies from one parental strain
520 (Nishizawa et al., 1999) and/or by PCR artefacts resulting from the use of non-proofreading
521 polymerases combined with high PCR cycle numbers. In any case, these sequences are very
522 closely related. Quite surprisingly, however, multiple infections with largely distinct strains
523 from the same genogroup occurred frequently, both in animals positive for only one
524 genogroup or in such positive for both (Fig. 3 B, large brackets to the right). The observed
525 extensive sequence variations of up to 15% (Fig. 4 B and data not shown) can not be
526 explained by the emergence of quasispecies or by PCR artefacts, but indicate the infection of
527 individual animals with distinct TTV strains of the same genogroup. Among the six
528 investigated sera that were tested positive for TTV1 and TTV2 by multiplex rt-PCR, two
529 animals were identified that carried clearly distinct strains from both genogroups, respectively
530 (Fig. 3 B, sera 8 and 13, and Fig. 4). Until lately, multiple infections with distinct TTV strains
531 from one genogroup have only been described for TTV1 and in one animal, but were not
532 properly discussed (Bigarré et al., 2005). However, very recently published findings from
533 others (Huang et al., 2009) confirm our results that multiple infections with distinct porcine
534 TTV1 and/or TTV2 strains occur. Extending these results, the observed frequency of multiple
535 infections with TTVs from both genogroups demonstrates that, similar to human TTV, such
536 multiple infections probably are common in pigs. Moreover, it appears that at least for the
537 relatively small sample number investigated, the occurrence of multiple infections is more
538 prominent for TTV2 than for TTV1 (Fig 3 B). Although this observation needs to be
539 statistically verified in future studies and the sequence analyses were not correlated to clinical
540 data, it still remains to be emphasized that multiple infections with distinct strains of the same
541 porcine TTV genogroup occurred quite frequently. This finding adds to the complex situation
542 that not only infections with pathogenic TTV strains that yet would need to be identified,
543 infections with (quasispecies of) one particular genogroup, double infections with both
544 genogroups, and/or their relative or absolute viral genomic loads might determine the

545 involvement of porcine TTV in disease induction. It now appears also possible that multiple
546 infections with distinct strains from the same or from both genogroups might represent a
547 relevant factor in disease induction. For human TTV, amino acid substitutions in
548 hypervariable regions of the capsid proteins of TTV quasispecies contribute to evasion of the
549 host's immune surveillance and favour the establishment of persistent infections (Nishizawa
550 et al., 1999). Similarly, the simultaneous presence of several related but distinct porcine TTV
551 strains might favour their immune evasion, establishment of infection, and eventually the
552 induction of disease. Therefore, future investigations should also focus on determining
553 whether and to which extent sequence variations or multiple TTV infections occur in diseased
554 animals. In this context it is to be mentioned that direct sequencing of genogroup-specific
555 PCR products (Martelli et al., 2006; Kekarainen et al., 2006; Martínez et al., 2006; Segalés et
556 al., 2009) is not appropriate, since in the case of multiple infections with strains from the
557 same porcine TTV genogroup the obtained data might merely represent a consensus sequence
558 of distinct strains present in the same sample. Instead, sequence analyses should include
559 several molecular clones of the obtained PCR products as described previously (Bigarré et al.,
560 2005; Brassard et al., 2008).

561 Taken together, both the newly established rt-PCR as well as previously described and
562 used methodology comprising conventional PCRs, molecular cloning, sequencing, and
563 phylogeny are valuable tools for a meaningful and continuative research on porcine TTV. In
564 answering urgent questions concerning (i) the influence of relative or absolute genogroup-
565 specific viral genomic loads during infection and (ii) the role of multiple infections with
566 distinct strains from one or both genogroups, they will help to elucidate the mechanism of
567 porcine TTV infection in the induction of disease.

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689 **8. Tables**

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691 **Table 1.** Results from multiplex real-time PCR analyses of sera from domestic pigs.

MULTIPLEX REAL-TIME PCR RESULTS					
	pigs	TTV1 - TTV2 -	TTV1 + TTV2 -	TTV1 - TTV2 +	TTV1 + TTV2 +
total	203	40	64	34	65
%	100	19.7	31.5	16.8	32

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693 Multiplex real-time PCR analyses specific for porcine Torque teno viruses of genogroup 1
 694 (TTV1) or genogroup 2 (TTV2). 203 serum samples were analyzed in two replicates,
 695 respectively. + ; both replicates positive. - ; one or both replicates negative.

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712 **Table 2.** Results from multiplex real-time PCR analyses of lung samples from wild boars.

MULTIPLEX REAL-TIME PCR RESULTS				
	TTV1 - TTV2 -	TTV1 + TTV2 -	TTV1 - TTV2 +	TTV1 + TTV2 +
%	12	20	49	19

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714 Multiplex real-time PCR analyses specific for porcine Torque teno viruses of genogroup 1
 715 (TTV1) or genogroup 2 (TTV2). 100 Lung samples were analyzed in three replicates,
 716 respectively. + ; two or three replicates positive. - ; two or three replicates negative.

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735 **Table 3.** Viral genomic loads of TTV-infected domestic pigs per ml serum.

	TTV1+ (n = 26)	TTV2+ (n = 44)	TTV1+/TTV2+ (n = 44)	
			TTV1	TTV2
min	$10^{2.86}$	$10^{2.53}$	$10^{3.64}$	$10^{2.49}$
max	$10^{5.88}$	$10^{7.39}$	$10^{7.06}$	$10^{6.66}$
mean	$10^{4.37}$	$10^{4.14}$	$10^{4.95}$	$10^{4.48}$
SD	$10^{0.82}$	$10^{1.19}$	$10^{0.94}$	$10^{1.17}$

736 Total number of samples analyzed by rt-PCR, 133. SD, standard deviation.

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757 **Table 4.** Sequence identities of TTV full-length sequences.

	TTV1 Sd-TTV31	TTV1 1p	TTV1 #471819	TTV2 2p	TTV2 #472142	dog Cf-TTV10
TTV1 Sd-TTV31	100	69.1	68.3	45.2	43.8	37.7
TTV1 1p	68.9	100	85.5	46.4	44.6	39.8
TTV1 #471819	67.9	85.2	100	47.6	45.4	38.9
TTV2 2p	43	44.2	45.4	100	85.9	37.2
TTV2 #472142	42.7	43.5	44.5	88	100	36.6
dog Cf-TTV10	36.6	38.7	38	38	36.6	100

758 Sequence identities are given as percentages of the strains to the left.

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779 **9. Figure captions**

780

781 **Fig. 1.** Sequence alignment of porcine Torque teno viruses (TTV). Reference strains 1p (Niel
 782 et al., 2005), TTV1 Sd-TTV31 (Okamoto et al., 2002), and 2p (Niel et al., 2005) are aligned
 783 in their untranslated regions. Deletions are indicated by hyphens. Nucleotide positions to the
 784 right refer to the respective published sequences (accession numbers AY823990, AY823991,
 785 and AB076001). Names and sequences (probes and sense primers, s) or binding sites (anti-
 786 sense primers, as) of primers and probes established for real-time (rt) and conventional PCR
 787 analyses performed in this study are highlighted. Oligonucleotides *TTV1 s* and *TTV2 s* have
 788 been described previously (Segalés et al., 2009).

789

790 **Fig. 2.** Agarose gel electrophoreses of products from conventional PCRs specific for (A)
 791 porcine Torque teno virus genogroup 1 (TTV1) or (B) genogroup 2 (TTV2) strains. The
 792 numbers on top refer to samples from 19 animals tested by porcine TTV-specific multiplex
 793 real-time (rt)-PCR. WC, water control. NC, negative control. PC, positive control. MM,
 794 molecular size markers. For TTV1-specific PCR, primers *TTV1 s* and *panTTV as* were used,
 795 while for detection of TTV2 primers *TTV2 s* and *panTTV as* were used (see Fig. 1). Results
 796 from multiplex rt-PCR specific for TTV1 (MRT1) and TTV2 (MRT2) are given below the
 797 gels. nd, not done. +, positive in both replicates. -, negative in both replicates.

798

799 **Fig. 3.** Distance-based phylogenetic analysis of porcine Torque teno virus genogroup 1
 800 (TTV1) or 2 (TTV2) UTR sequences obtained from conventional PCR analyses (see Fig. 2).
 801 Names of sequences refer to the numbering of animals in Fig. 2 (first number) and the number
 802 of cDNA clones sequenced (second number), respectively. TTV1 (1p, Sd-TTV31) and TTV2
 803 (2p) reference strains as well as a canine TTV outgroup strain (dog Cf-TTV10-1 (Okamoto et
 804 al., 2002)) are highlighted by boxes. The same tree is displayed in (A) standard or (B, left)

805 topology layout and results from calculating the alignment of 54 TTV sequences by the
806 neighbour joining algorithm followed by bootstrap analysis in 1000 replicates. Bootstrap
807 values higher than 840 are indicated. **(B, right)**: Brackets each connect porcine TTV1 or
808 TTV2 sequences obtained from the same animal.

809

810 **Fig. 4.** Alignment of **(A)** distinct porcine Torque teno virus genogroup 1 (TTV1) and **(B)**
811 genogroup 2 (TTV2) UTR sequences obtained from conventional PCR analyses of serum 13
812 (see Figs. 2 and 3). Nucleotide numbering to the right refers to individual sequence lengths
813 without the primers used for PCR. Sequence differences are highlighted and indicated by
814 reduced conservation bars below the alignments. Identical nucleotides are indicated by dots in
815 the lower sequences. Sequence identities between the displayed viral genomic regions: 92.4 %
816 for (A), 85.1% for (B).

817

818 **Fig. 5.** **(A)** Genome organizations of porcine Torque teno virus strains TTV1#471819 (left)
819 and TTV2#471942 (right). Names of regions and ORFs refer to Okamoto et al., 2002. ORF3s
820 result from predicted splicing of the respective mRNAs (Okamoto et al., 2002; Niel et al.,
821 2005). **(B)** Distance-based phylogenetic analysis of TTV1#471819 and TTV2#471942
822 compared to TTV1 (1p, Sd-TTV31) and TTV2 (2p) reference strains based upon the full-
823 length genomic nucleotide sequences and performed by the neighbour joining algorithm
824 followed by bootstrap analysis in 1000 replicates.

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TTV1-SdTTV31 **TTV1 s** 80

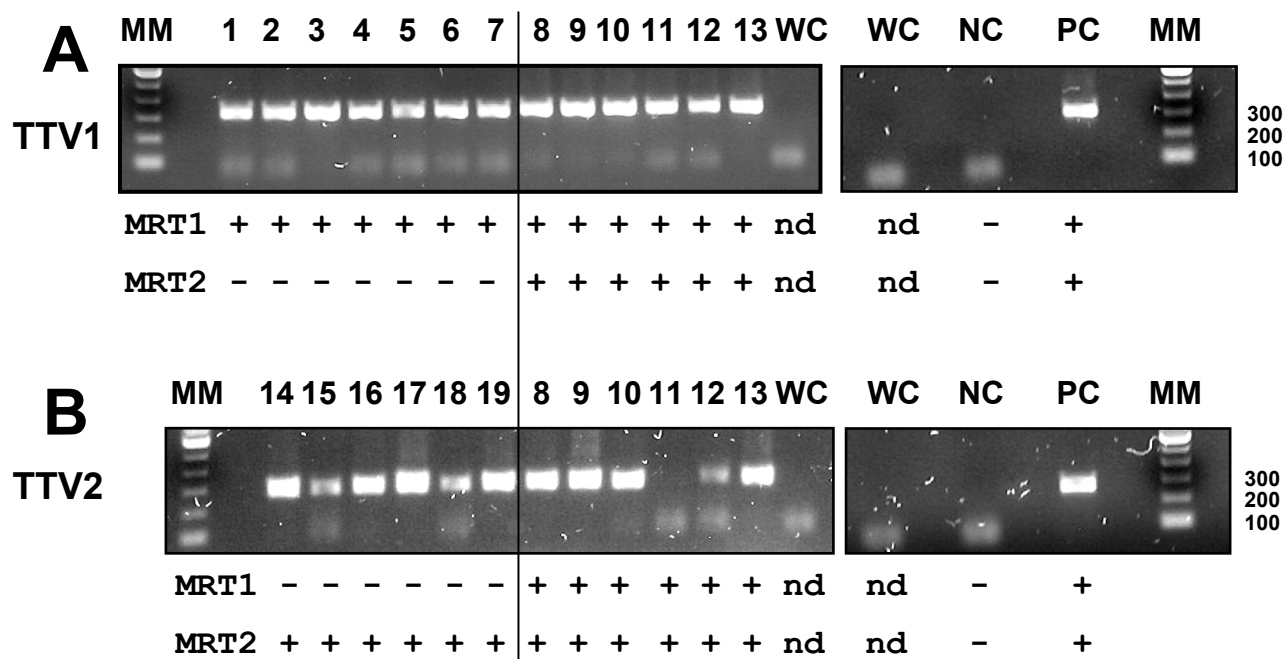
TTV 2p **TTV2 s** 66

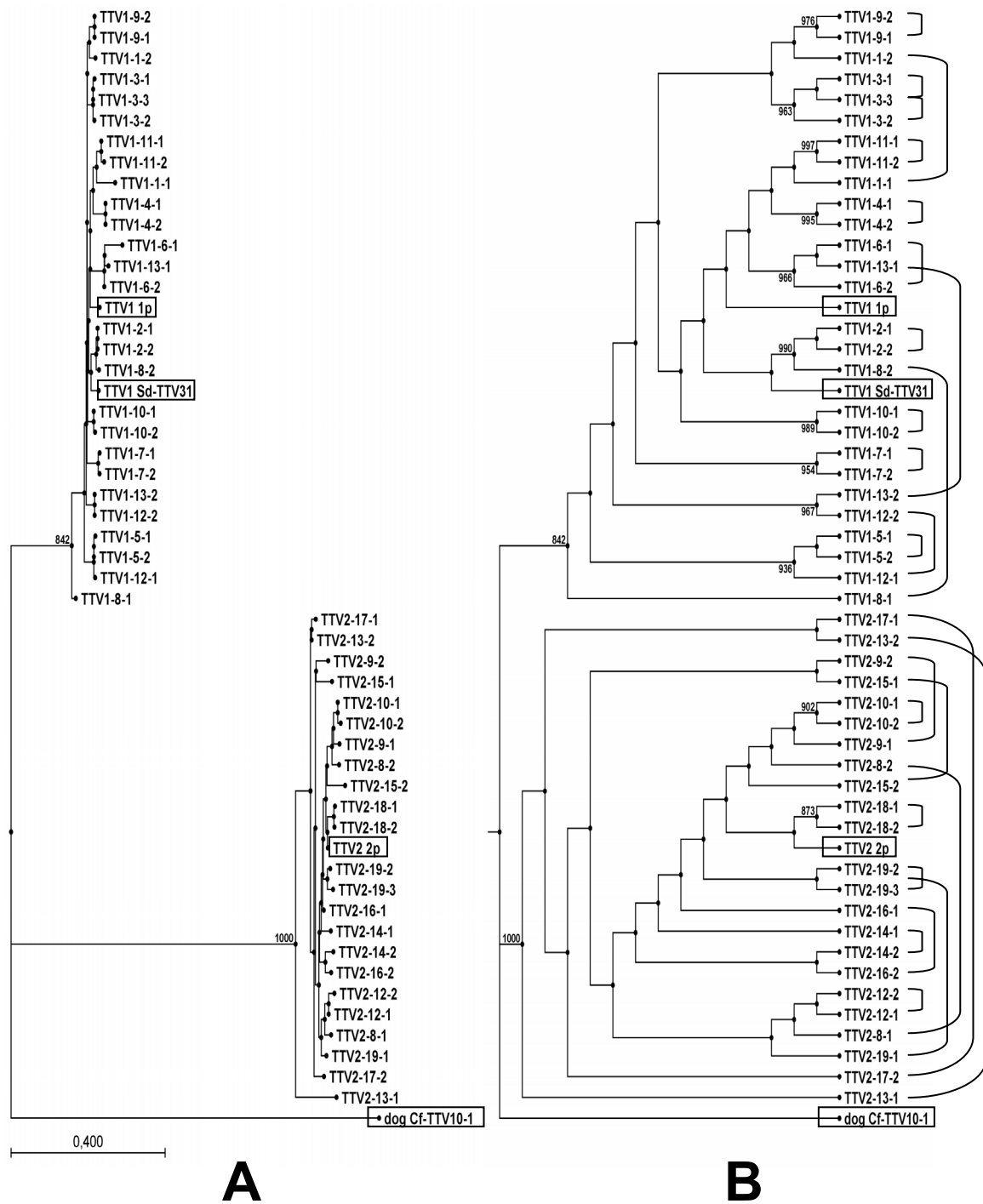
TTV 1p 353

TTV1-SdTTV31 **panTTV as** 353

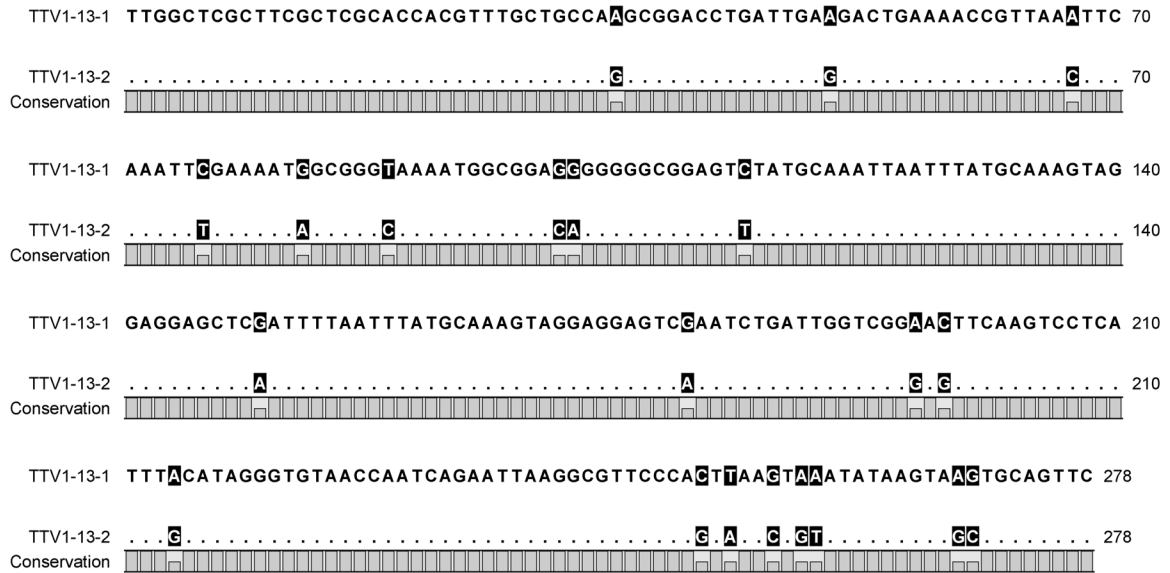
TTV 2p **rt-PCR TTV2 s** **TTV2 probe** **rt-PCR TTV2 as** 309

Accepted

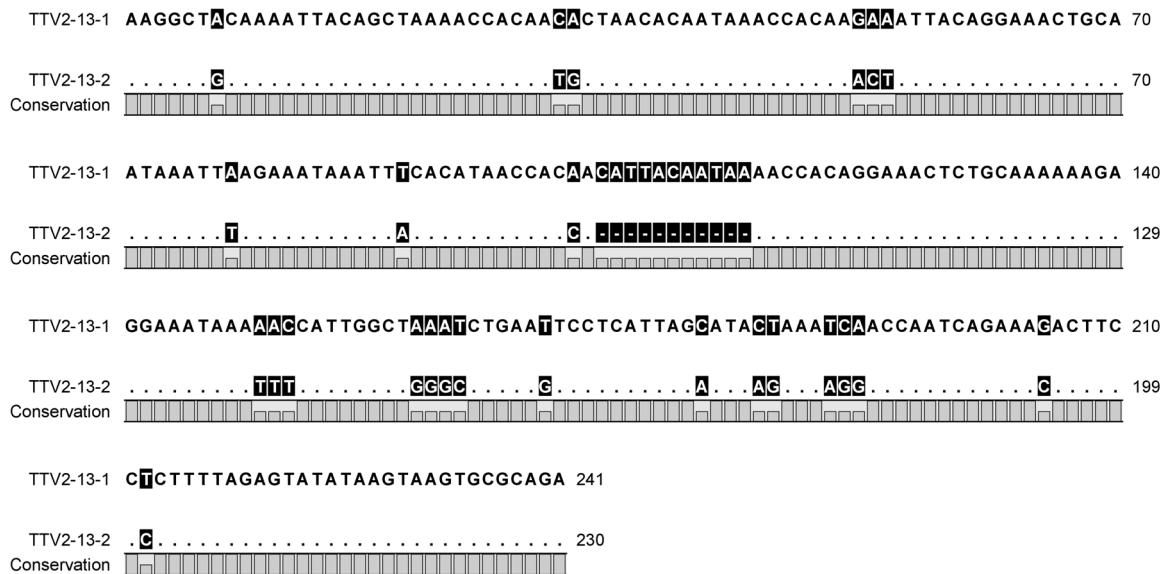


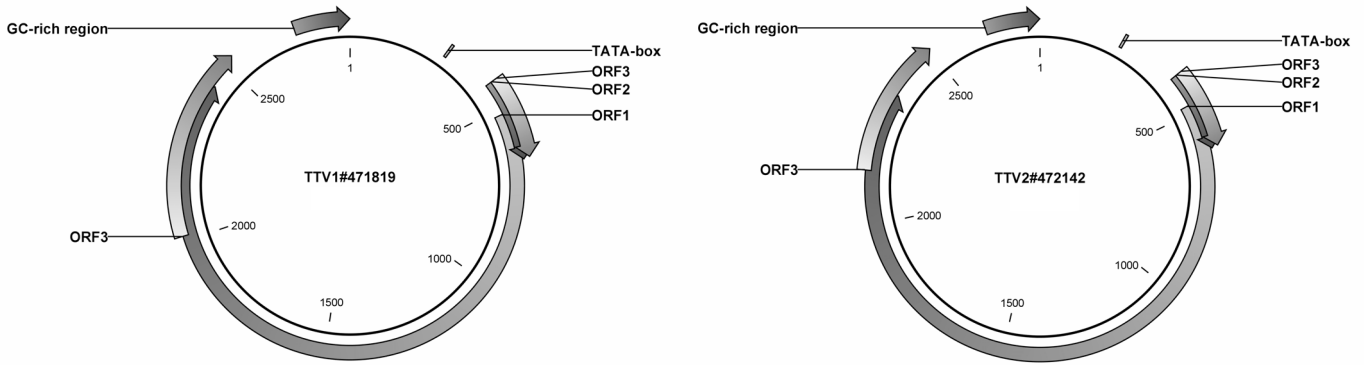


A



B



A**B**