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1 **Retrospective study on swine Torque teno virus genogroups 1 and 2 infection from**
2 **1985 to 2005 in Spain**

3

4 Joaquim Segalés^{1,2,*}, Laura Martínez-Guinó¹, Martí Cortey³, Nuria Navarro¹,
5 Eva Huerta¹, Marina Sibila¹, Joan Pujols^{1,4} and Tuija Kekkarainen¹

6

7 ¹Centre de Recerca en Sanitat Animal (CRESA), UAB-IRTA, Campus de la Universitat
8 Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain ²Departament de Sanitat i
9 Anatomia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona,
10 Spain

11 ³Bofill i Codina 14, Calella de P, (Girona), 17210, Spain

12 ⁴Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Barcelona, Spain

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14

15

16

17 ***Corresponding Author:** e-mail: joaquim.segales@uab.cat, Phone: +34 93 581 32 84

18 Fax: +34 93 581 44 90.

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25

26 **Abstract**

27 A retrospective study to detect evidence of swine Torque teno virus (TTV) genogroups 1
28 and 2 infection in sera of pigs from the Spanish livestock between years 1985 and 2005
29 was carried out by means of PCR. Also, the molecular evolution of TTV genogroups 1 and
30 2 during the 20-year period studied using a 250-base sequence of the non coding region of
31 the viral genome was assessed. Both TTV genogroup genomes were found in pig sera from
32 the first year of study. Taking into account the whole study period, 113 out of 162 animals
33 (69.8%) were infected with one or the other genogroup, while 38 out of 162 pigs (23.5%)
34 were co-infected with both genogroups. Moreover, TTV genogroup 2 (90 out of 162,
35 55.6%) was significantly more prevalent than genogroup 1 (54 out of 162, 33.3%). The
36 non-coding region of swine TTV genome sequenced showed its adequacy as a molecular
37 marker in swine TTV. This study represents the earliest evidence of TTV infection in pigs
38 to date, 14 years before the initial description of this virus. Moreover, this is also the
39 earliest evidence of TTV infection in any species.

40

41 *Keywords:* Torque teno virus (TTV), pig, genogroup, retrospective, phylogeny, evolution

42

43 Introduction

44

45 Torque teno virus (TTV), a recently discovered virus in humans and several domestic and
46 wild animal species including swine (Kekarainen & Segalés, 2008), belongs to the
47 “floating” genus Anellovirus. TTV is a small (in swine 2.8 kb) non-enveloped virus which
48 contains a single-stranded, negative sense, circular DNA genome. Two distinct TTV
49 genogroups have been identified so far in the domestic pig and wild boar (Niel et al., 2005;
50 Martínez et al., 2006). Detection of genogroup 1 in serum samples from domestic pigs of
51 different geographic regions (Canada, China, Korea, Spain, France, Italy, Thailand and
52 USA) revealed a prevalence ranging from 33% to 100% (Bigarré et al., 2005; McKeown et
53 al., 2004; Martelli et al., 2006; Brassard et al., 2007). The prevalence of genogroup 2 is
54 much lesser known and it has been addressed in a case-control study of postweaning
55 multisystemic wasting syndrome (PMWS), a disease caused by porcine circovirus type 2
56 (PCV2) (Segalés et al., 2005), in Spain, indicating an overall prevalence of 77%
57 (Kekarainen et al., 2006). Moreover, TTV genogroup prevalences in adult pigs has been
58 also preliminarily studied (Kekarainen et al., 2007); the prevalence of genogroup 1 in sera
59 and semen of adult boars were 64% and 55%, respectively, while a lower prevalence of
60 genogroup 2 was observed in both sera (38%) and semen (32%). Finally, prevalence of
61 swine TTV genogroups 1 and 2 in the wild boar were 58% and 66%, respectively
62 (Martínez et al., 2006). Therefore, it is nowadays believed that swine TTV genogroups are
63 widely spread, probably ubiquitous, in the domestic pig and wild boar (Kekarainen and
64 Segalés, 2008).

65

66 It has been suggested that human TTV is associated with different pathologies like
67 autoimmune rheumatic diseases (Gergely et al., 2006), liver pathologies (Kasirga et al.,

68 2005, Mushahwar et al., 1999, Nishizawa et al., 1997) and respiratory conditions (Biagini
69 et al., 2003, Maggi et al., 2003). However, there are no definitive data indicating that TTV
70 is responsible for any specific human disease. In the case of the pig, TTV infects a
71 relatively high proportion of animals that are apparently healthy (Kekarainen & Segalés,
72 2008). Therefore, it seems that TTV infection by itself does not represent a disease status.
73 However, its role during co-infection with other pathogens has not been investigated
74 thoroughly. Indeed, in the only published study of co-infection so far, a significantly higher
75 prevalence of swine TTV infection was found in sera from PMWS affected animals (97%)
76 compared to non-affected animals (78%) (Kekarainen et al., 2006). While PMWS affected
77 pigs (91%) were more likely to be infected with TTV from genogroup 2 than non-affected
78 swine (72%), no such difference was observed with genogroup 1.

79
80 Several laboratorial tests are useful to retrospectively detect the evidence of a viral
81 infection (Rodríguez-Arrijo et al., 2003). Firstly, serologic studies in archived sera may
82 give clues about the time of introduction of a virus in a given human or animal population.
83 Secondly, techniques to detect the antigen or the nucleic acid of the agent in formalin-
84 fixed, paraffin-embedded tissues, such as immunohistochemistry and *in situ* hybridisation,
85 respectively, can be applied to archived paraffin blocks. Finally, virus isolation or other
86 viral detection techniques such as polymerase chain reaction (PCR) from archived sera or
87 frozen/paraffin-embedded tissues would allow detecting as well as characterising the virus.

88
89 Taking into account the nowadays limitations of viral detection techniques available for
90 swine TTV (only PCR) and the relatively high or very high prevalence of this viral
91 infection in domestic swine (Kekarainen & Segalés, 2008) it was considered that PCR was
92 a fairly safe method to get reliable results on archived sera. Therefore, the purpose of the

93 present study was to perform a retrospective study to detect evidence of swine TTV
94 genogroups 1 and 2 infection in sera of pigs from the Spanish livestock between years
95 1985 and 2005. Moreover, a second objective was to describe the molecular evolution of
96 TTV genogroups 1 and 2 during the 20-year period studied based on partial sequences of
97 the non-coding region of the viral genome.

98

99

100 **Materials and methods**

101

102 ***Retrospective animal sera.***

103 One hundred and sixty two pig sera corresponding to 99 non-related Spanish farms
104 sampled between 1985 and 2005 were used for this study. Specifically, sera corresponded
105 to 89 postweaning pigs between 1 and 5 months of age (postweaning pigs) and 73 to adult
106 animals (sows); a maximum of 8 pig sera per year and per age-group were analysed (Table
107 1). Sera were not available for years 1992, 1993, 1998 and 1999. Samples were stored
108 frozen at -80°C until analysed. Sera corresponded to animals from different non-related
109 epidemiological studies performed across years and no information about the clinical status
110 of the individual studied pigs was available.

111

112 ***Polymerase chain reaction (PCR) to detect swine TTV genogroups.***

113 DNA from serum samples was extracted using a Nucleospin Blood DNA extraction kit
114 (Nucleospin® Blood, Macherey Nagel). Presence or absence of TTV genogroups 1 and 2
115 in serum samples was determined using specific, one-step PCR methods to amplify a
116 partial sequence (250 bases) of the non-coding region of swine TTV. Briefly, for TTV
117 genogroup 1, 20 µl of the PCR reaction contained 4 µl of serum DNA, 10 pmol of forward

118 primer-TTV1 (5'-CGGGTTCAGGAGGCTCAAT-3') and reverse primer-TTV1 (5'-
119 GCCATTCGGAAGTGCCTTACT -3'), 2.5 mM dNTPs, 2 mM of MgCl₂ and 0.75 U of
120 GoTaq® DNA Polymerase (Promega). The amplification was performed using a
121 GeneAmp® PCR System 9700 machine (Applied Biosystems, USA) and was initiated by
122 heating for 5 min at 94°C, followed by 50 cycles of 15 s at 94°C, 20 s at 54°C, 30s at 72°C
123 and a final extension for 5 min at 72°C. For TTV genogroup 2, amplification was carried
124 out as described above using primer pairs forward-TTV2 (5'-
125 TCATGACAGGGTTCACCGGA- 3') and reverse-TTV2 (5'-
126 CGTCTGCGCACTTACTTATATACTCTA- 3'). Finally, for each TTV genogroup, 15 µl
127 of PCR product was run on 1.8 % TAE-agarose gel. All PCR procedures

128

129 ***Statistical analyses on TTV genogroup prevalences.***

130 The average prevalences of TTV genogroups were compared globally, between pigs and
131 sows, between single-infected (one or other TTV genogroup) and co-infected (by both
132 TTV genogroups) animals, and among and within studied periods (1985-90, 1991-2000
133 and 2001-05) using Chi square (χ^2) tests. Significance was set at $P < 0.05$.

134

135 ***TTV genogroup sequencing.***

136 One or 2 PCR positive samples were selected for sequencing for each TTV genogroup and
137 available year. Amplified products were excised from the 1.8% agarose gel and purified
138 using QIAquick gel extraction kit (Qiagen GmbH). Sequencing reaction on both strands of
139 PCR products were done using Big Dye Terminator v3.1 cycle sequencing Kit (Applied
140 Biosystems) and run using the ABI Prism 3100 sequence analyser (Perkin Elmer).
141 Sequences were edited using VectorNTI and aligned with Clustal W programs using
142 AB076001 and AY823991 as reference sequences for genogroups 1 and 2, respectively.

143

144 ***Phylogenetic utility of TTV sequences.***

145 In order to evaluate the phylogenetic utility of the non-coding region of swine TTV,
146 several aspects of its evolution were analyzed. Firstly, the loss of phylogenetic information
147 due to substitution saturation and several measures of diversity (nucleotide diversity, mean
148 number of mutations per sequence and mean distance within and between genogroups)
149 were evaluated. The level of saturation was studied by plotting the pairwise number of
150 observed transitions and transversions versus genetic distance. In addition, substitution
151 saturation was evaluated with Xia's test (Xia et al., 2003). All these studies were
152 performed using the DAMBE program (Xia & Xie, 2001). Secondly, we calculated Fu &
153 Li's test (Fu & Li, 1993) and Tajima's D (Tajima, 1989) to explore if the DNA sequences
154 were evolving under a neutral model of selection with the DNAsp4.10 program (Rozas et
155 al., 2003). Thirdly, to avoid loss of power and accuracy in phylogenetic estimations (Bos &
156 Posada, 2005), the DNA substitution model that fitted available data best was explored
157 using ModelTest v3.8 (Posada & Crandall, 1998).

158

159 ***Phylogenetic analyses.***

160 Phylogenetic relationships among TTV genogroups were analyzed using Neighbor-Joining
161 (NJ), Maximum Likelihood (ML) and Maximum Parsimony (MP) inference methods as
162 described by Olvera et al. (2007). Additionally, sequences were compared by Bayesian
163 Inference (BI) with Mr. Bayes 3.1 (Huelsenbeck & Ronquist, 2001; Ronquist &
164 Huelsenbeck, 2003) constructing two Monte-Carlo chains during 400000 generations,
165 evaluating the log-likelihoods and re-sampling each chain every 40 generations; then, a
166 tree with clade credibility was constructed using the posterior probability distribution
167 calculated from the log-likelihoods during chain construction. Trees were rooted using

168 three sequences from primate TTVs (AB041958, AB041959 and AB041961). Finally, we
169 estimated a maximum parsimony network of the non-coding region of swine TTV by the
170 statistical parsimony method of Templeton et al. (1992) using the TCS computer program
171 (Clement et al., 2000).

172

173

174 **Results**

175

176 *Detection of TTV genogroups on retrospective sera.*

177 Both TTV genogroup genomes were found by PCR in pig sera from the first year of study,
178 and almost found in all examined years (Table 1). Taking into account the whole study
179 period, 113 out of 162 animals (69.8%) were infected with one or the other genogroup,
180 while 38 out of 162 pigs (23.5%) were co-infected with both genogroups. The percentage
181 of pigs co-infected with both TTVs was not different for both age-groups (14 out of 73,
182 19.8%, in sows, and 23 out of 89, 25.8%, in postweaning pigs). Moreover, TTV genogroup
183 2 (90 out of 162, 55.6%) was more prevalent than genogroup 1 (54 out of 162, 33.3%); this
184 result was also significant when postweaning pigs were considered as a separated group,
185 but not for sows.

186

187 No significant differences in prevalence of TTV genogroups 1 and 2 were observed among
188 decades. Within a given decade, no significant differences between TTV genogroup
189 prevalences were observed, but 1991-2000; a higher prevalence of genogroup 2 ($p < 0.05$)
190 was observed in postweaning pigs as well as overall (considering all age-groups together)
191 for that period. No differences within each TTV genogroup prevalence among age-groups

192 were observed for initial studied decades, but both genogroups were significantly more
193 prevalent ($p < 0.05$) in postweaning pigs during the 2001-05 period.

194

195 ***Evaluation of TTV non-coding region as a molecular marker.***

196 Saturation effects were investigated plotting the absolute number of transitions and
197 transversions versus genetic distance (Fig. 1A and 1B). The number of observed
198 transversions relative to that of transitions gradually increased with growing divergence,
199 and both data sets resembled a line, indicating that transitions and transversions were not
200 saturated. Moreover Xia's test supported little saturation for TTV genogroups 1 and 2 (Iss
201 $< \text{Iss.c}$, $p < 0.0005$). Besides, a neutral model of selection for the DNA segment was
202 supported by the non significant results of Tajima's D and Fu & Li's test, for the entire
203 dataset, and for genogroups 1 and 2 separately. The levels of nucleotide diversity per site
204 between two sequences (0.0468 vs 0.05747) and mean number of substitutions per
205 sequence (11.207 vs 12.037) were slightly higher in genogroup 2, as well as mean
206 distances within genogroups (0.0572 ± 0.0090 vs 0.0644 ± 0.0103). Finally the complete
207 dataset and both genogroups separately presented a biased pattern of mutation, as indicated
208 by gamma parameters below zero ($\alpha_{\text{all}}=0.4050$, $\alpha_1=0.7600$, $\alpha_2=0.6783$).

209

210 ***Phylogenetic relationships between TTV genogroups.***

211 Figure 2 shows one of the phylogenetic trees assayed (NJ method), even though ML, MP
212 and BI trees were very similar and shared features of evolutionary relevance. In all trees,
213 strains identified as the same genogroup according with the sequence synapomorphies
214 were placed together and no discrepancies among inference algorithms were reported.
215 Thus, two monophyletic clusters were resolved in each inference method corresponding to
216 genogroups 1 and 2. Additionally, statistical parsimony networks for both genogroups

217 showed a dispersal distribution of age groups along the network (Fig. 3A and B),
218 indicating no relationship between the date of virus detection and the clusters in which
219 strains were found.

220

221

222 **Discussion**

223

224 Data compiled in this retrospective study indicate that both swine TTV genogroups have
225 been circulating at least since 1985 in the Spanish pig population, fourteen years before the
226 first description of TTV existence in pigs (Leary et al., 1999). Therefore, this study
227 represents the earliest evidence of TTV infection in pigs to date. Moreover, this is also the
228 earliest evidence of TTV infection in any species; since the discovery of TTV in humans in
229 1997 (Nishizawa et al., 1997), only one work indicated the existence of previous TTV
230 infection, specifically in patients that underwent cardiovascular surgery and blood
231 transfusion between 1991 and 1992 (Yang et al., 2000). It is important to note, also, that no
232 apparent swine TTV genogroup prevalence variation was observed during the study period,
233 since percentage of infected animals across time was fairly similar.

234

235 Taking into account that 4 out of 5 adult pigs and 2 out of 5 postweaning pigs were already
236 infected by TTV1 or TTV2 in the very first year of study, it is obvious that those viruses
237 were already widespread in 1985. The introduction of this virus in the Spanish pig
238 livestock had to occur sometime previously to the initial year of study and, therefore, TTV
239 in pigs should not be considered as a true new emerging virus, since it has remained
240 unnoticed for a long period of time. This situation is fairly equivalent to that of other pig
241 viruses considered initially as emergent, in which retrospective studies have shown

242 evidence of their infection much before its first description. Porcine circovirus type 2
243 (PCV2), the essential infectious agent of postweaning multisystemic wasting syndrome
244 (PMWS) (Segalés et al., 2005), was initially described in 1998 (Hamel et al., 1998;
245 Meehan et al., 1998), but retrospective studies showed evidence of PCV2 infection as soon
246 as 1962 in Europe (Kruger et al., 2004), 1973 in America (Ramírez-Mendoza et al., 2008)
247 and 1989 in Asia (Mori et al., 2000). Moreover, PMWS was also unnoticed for a number
248 of years, since retrospective studies showed cases fulfilling disease case definition at least
249 by 1985 (Kruger et al., 2004). Similarly, swine hepatitis E virus, described initially in 1997
250 (Meng et al., 1997), has been shown in pigs as early as 1995 in Asia (Jung et al., 2007) and
251 1985 in Europe (M. Casas, CReSA, Spain, personal communication). A common fact in all
252 these retrospective works was that first evidence of viral infection coincided with the very
253 first year of study and, therefore, it implied also the assessment of a widespread nature of
254 these viral infections since then as well. The lack of very old pig serum or tissue samples
255 impedes to date the emergence of viruses like TTV, HEV and PCV2. Porcine reproductive
256 and respiratory syndrome virus (PRRSV), which caused an emerging pig disease by
257 middle eighties in North-America and early nineties in Europe and one of the most
258 important swine diseases worldwide (Rossow, 1998), would be an exception to the
259 previously mentioned viral agents. Evidence of PRRSV infection was recorded in 1979 in
260 North-America (Carman et al., 1995) and 1988 in Europe (Ohlinger, 1992), relatively close
261 to its emergence as an overt disease.

262

263 The evaluation of the TTV non-coding region as a molecular marker to detect differences
264 between and within TTV genogroups gave clear results: the selected DNA fragment was
265 not saturated, nor under selection pressure, and the molecular diversity was enough to

266 detect differences between and within genogroups. Overall, the analyses performed on the
267 non-coding region showed its adequacy as a molecular marker in swine TTV.

268

269 Importantly, statistical parsimony networks for both genogroups showed a dispersal
270 distribution of both genogroups along the network. This result suggests that, from a
271 phylogenetical point of view, TTV genogroups 1 and 2 have not changed between 1985
272 and 2005, since no relationship between the date of virus detection and the phylogenetic
273 clusters were found. It must be emphasized, however, that although the TTV non-coding
274 region is a good molecular marker, the sequence studied represents less than 10% of the
275 total genome (Kekarainen & Segalés, 2008). It cannot be ruled out that other regions of the
276 swine TTV genome, presumably under higher mutation pressure (i.e., ORF1, which
277 encodes the capsid protein), would be more useful to assess phylogenetic evolution, as it
278 has been suggested for PCV2 (Olvera et al., 2007; Dupont et al., 2008), a virus similar to
279 TTV. Further work on other swine TTV sequences (ORF1, 2 and 3) or the whole genome
280 would be desirable for phylogenetic studies.

281

282 The current study does not allow assessing the potential involvement of swine TTV in pig
283 diseases or pathological conditions. Studied sera came from different epidemiological
284 studies performed along 20 years and the specific health status of each animal was not
285 recorded. However, based on the nature of the original studies, it is very probable that a
286 significant number of sera corresponded to healthy animals. If swine TTV may interact
287 with other pathogens to cause clinical disease remains to be elucidated.

288

289 In conclusion, data of the present study indicate that infection of different age-group pigs
290 by swine TTV genogroups 1 and 2 was already widespread in 1985, suggesting that the
291 introduction of this virus in the Spanish livestock occurred previously. In addition, the
292 TTV non-coding region was found useful as a molecular marker for phylogenetic studies,
293 although no relationship between the date of virus detection and the phylogenetic clusters
294 was found.

295

296

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298

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301

302

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421 **Figure legends**

422

423 **Fig. 1.** Plot representation of the number of transitions (×) and transversions (Δ) *versus* the
424 genetic distance calculated with the Tamura-Nei model among all combinations of
425 pairwised strains of TTV genogroups 1 and 2. Solid lines indicate the best fit to the
426 observed data in each mutational type.

427

428 **Fig. 2.** Phylogenetic tree based on the NJ method for the 41 sequences plus 3 outgroups
429 used in the study using the Hasegawa-Kishino-Yano substitution rate, and considering a
430 gamma parameter of 0.405. Only bootstrap support values higher than 50% are shown.
431 ML, MP and BI inference methods presented a very similar branching pattern. AB076001
432 and AY823991 represent suggested reference strains for genogroup 1 and 2, respectively.
433 Age groups: ■ 1985-1990, ● 1991-2000 and ◆ 2001-2005.

434

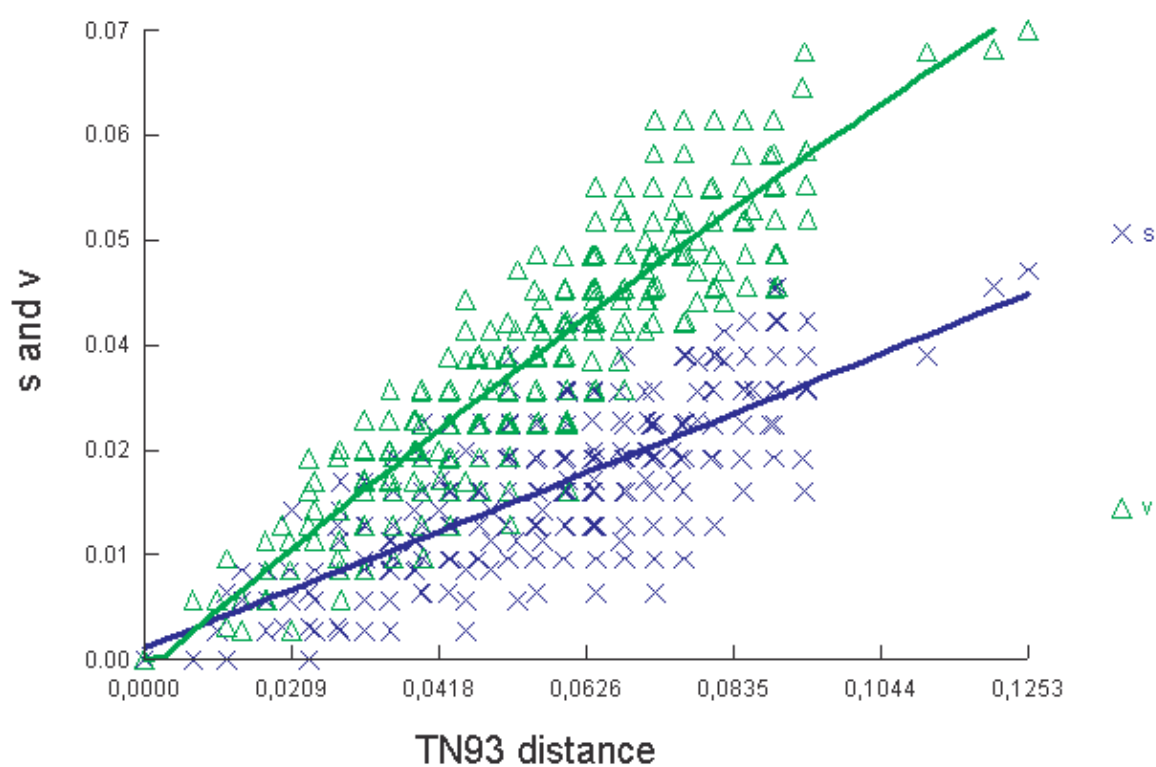
435 **Fig. 3.** Statistical parsimony networks for genogroups 1 (A) and 2 (B). Numbers along
436 branches represent the number of mutations that separate every node/strain, mutations are
437 showed beside. AB076001 and AY823991 represent suggested reference strains for
438 genogroup 1 and 2, respectively. Age groups as described in Fig.2.

1 Table 1
 2 Prevalence of swine TTV genogroups 1 and 2 by PCR in pigs in different years and age-
 3 groups (adults and postweaning pigs).

Year	Group of age	No. of tested sera	TTV genogroup 1*	TTV genogroup 2*	TTV genogroups 1 and 2*	TTV genogroups 1 or 2*
1985	Adults	5	1	4	1	4
	Postweaning	5	1	2	1	2
1986	Adults	5	1	2	0	3
	Postweaning	5	1	2	1	2
1987	Adults	5	1	4	1	4
	Postweaning	5	2	3	1	4
1988	Adults	5	3	3	1	5
	Postweaning	8	5	5	4	6
1989	Adults	5	3	4	2	5
	Postweaning	5	2	3	1	4
1990	Adults	5	3	1	0	4
	Postweaning	8	3	8	3	8
1991	Adults	5	4	4	3	5
	Postweaning	5	0	1	0	1
1994	Adults	5	0	0	0	0
	Postweaning	5	0	2	0	2
1995	Adults	5	0	0	0	0
	Postweaning	5	1	3	0	4
1996	Adults	5	1	2	1	2
	Postweaning	5	1	3	1	3
1997	Adults	5	4	4	3	5
	Postweaning	5	2	4	2	4
2000	Adults	5	1	2	0	3
	Postweaning	5	1	3	1	3
2001	Adults	3	1	0	0	1
	Postweaning	3	2	3	2	3
2002	Adults	0	NT	NT	NT	NT
	Postweaning	5	2	3	1	4
2003	Adults	5	1	3	1	3
	Postweaning	5	3	5	3	5
2004	Adults	5	1	1	1	1
	Postweaning	5	1	2	0	3
2005	Adults	0	NT	NT	NT	NT
	Postweaning	5	2	4	2	4
TOTAL	Adults	73	25	34	14	45
	Postweaning	89	29	56	23	68
	Total	162	54	90	38	113

4 * No. of positive sera by PCR; NT = not tested

Genogroup 1



Genogroup 2

