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1 **Development and validation of a real-time two-step RT-qPCR**

2 **TaqMan® assay for quantitation of Sacbrood virus (SBV) and its**

3 **application to a field survey of symptomatic honey bee colonies**

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Abstract

Sacbrood virus (SBV) is the causal agent of a disease of honey bee larvae, resulting in failure to pupate and causing death. The typical clinical symptom of SBV is an accumulation of SBV-rich fluid in swollen sub-cuticular pouches, forming the characteristic fluid-filled sac that gives its name to the disease. Outbreaks of the disease have been reported in different countries, affecting the development of the brood and causing losses in honey bee colonies. Today, few data are available on the SBV viral load in the case of overt disease in larvae, or for the behavioural changes of SBV-infected adult bees. A two-step real-time RT-PCR assay, based on TaqMan® technology using a fluorescent probe (FAM-TAMRA) was therefore developed to quantify Sacbrood virus in larvae, pupae and adult bees from symptomatic apiaries. This assay was first validated according to the recent XP-U47-600 standard issued by the French Standards Institute, where the reliability and the repeatability of the results and the performance of the assay were confirmed. The performance of the qPCR assay was validated over the 6 log range of the standard curve (*i.e.* from 10^2 to 10^8 copies per well) with a measurement uncertainty evaluated at 0.11 log10. The detection and quantitation limits were established respectively at 50 copies and 100 copies of SBV genome, for a template volume of 5 µl of cDNA. The RT-qPCR assay was applied during a French SBV outbreak in 2012 where larvae with typical SBV signs were collected, along with individuals without clinical signs. The SBV quantitation revealed that, in symptomatic larvae, the virus load was significantly higher than in samples without clinical signs. Combining quantitation with clinical data, a threshold of SBV viral load related to an overt disease was proposed (10^{10} SBV genome copies per individual).

Keywords: Sacbrood virus (SBV), Real-time RT-PCR, Validation, Field survey, *Apis mellifera*.

2

1. Introduction

Sacbrood is an infectious disease affecting the larvae of the honey bee causing characteristic clinical symptoms in developing broods. Larvae with sacbrood fail to pupate, and ecdysial fluid rich in sacbrood virus (SBV) accumulates beneath their unshed skin, forming the sac after which the disease is named (Bailey et al., 1964; Ball and Bailey, 1997). Sacbrood virus was the first honey bee virus to be identified (White, 1917) and the first for which the genome organisation and the complete nucleotide sequence were determined (Ghosh et al., 1999). It is a positive-sense single-stranded-RNA virus of the *Picornavirales* order. It belongs to the *Iflavirus* genus, from the recently established family *Iflaviridae* (King et al., 2011).

SBV is widely distributed. It attacks *Apis mellifera* colonies in every continent (Allen and Ball, 1996). Generally it has no major consequences for colony survival. However, sacbrood is a major cause of colony mortality for *A. cerana* colonies in Asia (Liu et al., 2010). The prevalence of this virus has been investigated in various countries, such as Great Britain (Bailey, 1967), Australia (Dall, 1985), Austria, Germany, India, Nepal and South Africa (Grabensteiner et al., 2001), France (Tentcheva et al., 2004), the USA (Chen et al., 2004), Uruguay (Antunez et al., 2006), Denmark (Nielsen et al., 2008) and Spain (Kukielka and Sánchez-Vizcaíno, 2009). Although it is primarily a disease of larvae, SBV also multiplies in pupae and adult bees where it provokes physiological and behavioural changes that limit its transmission to larvae. This includes an accelerated progression from brood tending to foraging (Bailey and Ball, 1991), a degeneration of hypopharyngeal glands (Du and Zhang, 1985), and a strong aversion to eating or collecting pollen (Bailey and Fernando, 1972; Anderson and Gaicon, 1992). The behaviour of drones, which never eat pollen, appears unaffected by SBV infection (Ball and Bailey, 1997). Some authors have reported overt disease in brood (Allen and Ball, 1996; Grabensteiner et al., 2001; Nielsen et al., 2008), while others have reported SBV detection without clinical symptoms (Tentcheva et al., 2004; Antunez et al., 2006;

84 Kukielka and Sánchez-Vizcaíno, 2009). Although real-time RT-PCR detection methods

85 have been recently developed for the detection and the quantitation of SBV

86 (Chantawannakul et al., 2006; Gauthier et al., 2007; Kukielka and Sánchez-Vizcaíno,

87 2009; Lock et al., 2012; Yoo et al., 2012; Evison et al., 2012), few data are available on

88 the SBV viral titres of larvae samples presenting typical symptoms of SBV.

89 In this paper, a new real-time two-step RT-PCR based on the TaqMan® technology to

90 quantify SBV was described. This SBV RT-qPCR was then validated according to the XP

91 U47-600 French standard (as already described for chronic bee paralysis virus (CBPV)

92 by Blanchard et al., (2012)). Finally, this method was assessed on field samples collected

93 from an outbreak of sacbrood in France in 2012.

94 The development of this quantitative assay for SBV genomic RNA was performed using

95 TaqMan® PCR with minor groove binder (MGB) probe technology. A DNA probe with a

96 conjugated MGB group forms an extremely stable duplex with single-stranded DNA

97 targets, allowing the use of shorter probes. The shorter probe length endows MGB

98 probes with greater sensitivity and lower fluorescent background compared to non-MGB

99 probes (Kutyavin et al., 2000). The SBV RT-qPCR assay was validated for (i) the

100 analytical specificity, (ii) the PCR detection limit (DL_{PCR}), (iii) the PCR quantitation limit

101 (QL_{PCR}) and (iv) the linearity and efficiency of the qPCR assay. The method was then

102 applied to assess the SBV genomic load in different categories of samples (larvae with or

103 without typical SBV clinical symptoms, pupae and adult bees) coming from apiaries

104 suffering from sacbrood.

105

106 **2. Materials and methods**

107

108 2.1 Real-time quantitative RT-PCR method

109 The SBV method development was based on the TaqMan® two-step real-time

110 quantitative RT-PCR CBPV assay developed previously by Blanchard et al., (2012) using

111 the Applied Biosystems 7500 System (Applera). The SBV-specific primers and probe

4

112   were designed using the Primer Express 3.0 software (Applera). The choice of primers

113   and probe was constrained by absolute sequence conservation across all the SBV

114   sequences deposited in GenBank, including the Thai, Chinese and Korean variants

115   (Figure 1). The chosen primer set amplifies a 70 bp fragment located in the N-terminal

116   part of the polyprotein region of the SBV genome: nucleotides 434 to 503 according to

117   the reference sequence (GenBank accession no. AF092924, Ghosh et al., 1999). The

118   forward primer is SBV-F434: 5'- AACGTCCACTACACCGAAATGTC and the reverse

119   primer is SBV-R503: 5'- ACACTGCGCGTCTAACATTCC. The TaqMan® probe is SBV-

120   P460: 5'- TGATGAGAGTGGACGAAGA, labelled with the fluorescent reporter dye FAM

121   (6-carboxyfluorescein) at the 5' end and with the non-fluorescent quencher (NFQ) with

122   MGB at the 3' end.

123   The PCR is performed in duplicate in a MicroAmp optical 96-well reaction plate,

124   containing 1X Taqman® Universal PCR Master Mix with uracil-N-glycosylase (UNG -

125   Applied Biosystems), 320 nM of each primer (SBV-F434 and SBV-R503), 200 nM of the

126   qSBV probe, 1X Exo IPC Mix and 1X Exo IPC DNA from IPC VIC™ Probe (Applied

127   Biosystems). UNG reduces false-positive results by destroying contaminating, dUTP-

128   incorporated PCR products from previous runs. The Exo-IPC reagents are a pre-

129   optimized blend of an Exogenous Internal Positive Control (IPC) DNA template plus

130   corresponding primers and VIC-based probe, designed to distinguish true-negative

131   results from false-negative results due to PCR inhibition. The VIC-based signal of the IPC

132   amplification is detected separately from the FAM-based signal of the SBV amplification.

133   The reaction volume was completed with 5 µl of template, either 10^2 to 10^8 copies of the

134   SBV plasmid DNA standard (described below) or random-hexamer primed cDNAs,

135   obtained as previously described in Blanchard et al., (2007), giving a total volume of 25

136   µl. The thermal cycling conditions are 2 min at 50°C (active temperature for UNG

137   degradation of contaminating PCR-products), 10 min at 95°C (activation of AmpliTaq

138   Gold DNA Polymerase and inactivation of UNG), followed by 40 cycles of denaturation at

139   95°C for 15 s and annealing/extension at 60°C for 1 min. Results are expressed as the

140 mean of the two replicates of each reaction. A biological positive control is included in

141 each run, obtained from homogenate of symptomatic larvae. Two negative controls are

142 also included in each run: a blank RNA extraction negative control and a template-free

143 qPCR negative control, in which the sample is replaced by purified water.

144 .

145

146 2.2 Analytical specificity

147 Analytical specificity was assessed as described by Blanchard et al. (2012). The *in silico*

148 analysis was performed by a Blast search on the genome database with the 70-bp

149 sequence of the SBV amplicon. The experimental specificity was evaluated by exclusivity

150 and inclusivity tests, testing the assay's ability not to amplify any RNA that is not SBV

151 (exclusivity) and to positively detect all strains of SBV (inclusivity). The exclusivity test

152 was performed on cDNAs obtained from bee samples infected with other bee viruses,

153 such as ABPV, CBPV, IAPV, BQCV or DWV. The inclusivity test was carried out on

154 several SBV isolates from various geographic regions (Austria, Denmark, France,

155 Sweden and Uruguay), coming from apiaries with or without clinical symptoms and thus

156 constituting a panel of samples that best represents SBV genetic diversity. The genetic

157 diversity among these isolates was assessed by a phylogenetic analysis of the primer-

158 free sequences of a 469 bp amplicon located in the structural protein region,

159 encompassing the 70 bp fragment of the SBV assay, amplified by conventional PCR

160 using primers SBV-1f and SBV-2r described by Grabensteiner et al. (2001). The

161 sequences were aligned to homologous SBV sequences obtained from GenBank (Table

162 1) using CLUSTAL_X (Thompson et al., 1997). Phylogenetic trees were constructed

163 using the neighbour-joining (NJ), maximum likehood (ML) and maximum parsimony (MP)

164 methods as implemented by the PHYLOWIN program (Galtier et al., 1996). Statistical

165 support for the branching pattern was provided by bootstrap analysis involving 500

166 replicates. Branches with less than 50% bootstrap were omitted. The phylogenetic trees

167 were drawn using TreeView (Page, 1996).

169 2.3 Experimental scheme to assess the detection and quantitation limits of the SBV

170 qPCR assay

171 The detection and quantitation limits of the SBV qPCR (DL_{PCR} and QL_{PCR}) were

172 determined from a 4.44 kb plasmid obtained by cloning the 469 bp PCR fragment

173 amplified from the polyprotein region of SBV with primers SBV-1f and SBV-2r

174 (Grabensteiner et al., 2001) into a pCR® II TOPO® vector (Invitrogen). The plasmid DNA

175 concentration was determined by spectrophotometry (three trials on three independent

176 dilutions) and converted to plasmid copies per microliter. From this, stock solutions

177 containing 0.2×10^2 to 0.2×10^8 copies per microliter were prepared through 10-fold

178 serial dilutions in TE buffer. These stock solutions were used to establish the standard

179 calibration curves for absolute quantitation, for calculating the assay performance

180 parameters, and for estimating the assay detection limits. The experimental scheme was

181 conducted as described by Blanchard et al., (2012). To determine the qPCR detection

182 limit, three independent trials were performed on three independent two-fold serial

183 dilutions (200 to 6.25 DNA copies for 5 µl of template), with eight replicates of each

184 dilution level. The DL_{PCR} was determined by the smallest number of nucleic acid targets

185 given 95% of positive results. To assess the linearity domain and the quantitation limit,

186 three independent trials were performed on three independent 10-fold serial dilutions.

187 Standard curves were generated by linear regression analysis of the threshold cycle (C_T)

188 measured for each amplification *vs.* the $\log_{10}$ copy number for each standard dilution.

189 The measured quantity for each dilution level was determined in retrospect by using the

190 formula: $\log x = [C_T - b] / a$, where *a* is the slope, *b* is the Y-intercept, and x is the

191 quantity. The obtained values were compared to the theoretical quantities and provided,

192 for each load level, the mean bias (*mb*). The standard deviation of the obtained values

193 (*SD*) was determined, and the uncertainty of the linearity was calculated using the

194 formula $U_{LINi} = 2[\sqrt{SD^2 + mb^2}]$. The combined linearity uncertainty was defined for the

195 entire calibration range and given by the formula $U_{LIN} = |\sqrt{\Sigma U_{LINi}^2 / k}|$ where k is the

196 number of dilution levels. The quantitation limit of the assay is then determined by the first

197 level load of the calibration range.

198 To evaluate the performance of the entire method (RNA extraction to qPCR analysis), the

199 positive control, obtained by homogenisation of symptomatic larvae, was followed by

200 means of a control chart. In order to be accepted, the quantitative result had to be within

201 the acceptability limits. These limits were defined by the mean of the first ten values ±

202 twice the standard deviation.

203

204 2.4 Application of the SBV RT-qPCR method on bee samples from honey bee colonies

205 with SBV clinical symptoms

206 In 2012, clinical symptoms of SBV were observed in French apiaries by the "Brigade

207 Nationale d'Enquêtes Vétérinaires et Phytosanitaires" (BNEVP). Four apiaries located in

208 three French departments (Loiret, Lot et Garonne and Vendée) were visited in April 2012.

209 Three to five hives were sampled from each apiary. Samples of bee larvae showing

210 typical SBV clinical symptoms were collected (12 samples of 2 to 5 larvae per hive),

211 together with samples of asymptomatic larvae from the same hives (12 samples of 15

212 larvae per hive), samples of pupae (12 samples of 10 pupae per hive) and samples of in-

213 house bees (12 samples of 10 bees per hive). Sample processing, RNA extraction and

214 cDNA synthesis were performed as described in Blanchard et al. (2007). Five microliters

215 of the cDNA were used as template for the qPCR.

216

217 2.5 Statistical analysis

218 The SBV genomic loads obtained from the bee samples were analysed by the Two-

219 sample Kolmogorov-Smirnov and the Kruskal-Wallis non-parametric tests using the

220 SYSTAT 9 computer software package (SPSS Inc.).

221

222 **3. Results**

223

224   3.1 Analytical specificity

225   In order to assess the specificity of the SBV assay, the chosen 70 bp sequence was

226   searched *in silico* by a Blast analysis of the DNA public database. Only SBV sequences

227   were identified by this search. Experimentally non significant amplification was detected

228   in cDNAs obtained from ABPV, CBPV, IAPV, BQCV and DWV, which implies an

229   exclusive specificity.

230   The inclusivity test was carried out on different isolates from Austria, France, Denmark,

231   Sweden and Uruguay. A phylogenetic analysis was realised with isolates that gave a

232   positive result in conventional PCR. SBV sequences from GenBank were included in this

233   study. All the methods used for the inclusivity test (NJ, ML and MP) gave similar profiles.

234   Results using the ML method are shown in figure 2. This analysis (realised on sequences

235   of the SBV polyprotein region) revealed that all the European isolates (Austria, France,

236   Denmark and Sweden) are included in a group close to the UK and Uruguay genotypes,

237   whereas the Asian and Korean genotypes are included in an another group (Figure 2).

238   Afterwards, samples of bees, larvae, pupae or cDNA were tested by SBV qPCR. The

239   SBV genomic loads observed in adult bees and cDNAs were moderate, with 10^5 to 10^7

240   SBV copies per bee and 10^4 to 10^7 SBV copies per µl of cDNA. On the other hand, SBV

241   genomic loads observed in larvae and pupae samples were much higher, over 10^{11}

242   copies per individual.

243

244   3.2 Determination of the detection limit of the SBV qPCR assay

245   The detection limit of the SBV qPCR assay was determined using three independent 6-

246   step, 2-fold dilutions series of the 4.44 kb plasmid ranging from 200 to 6.25 DNA copies

247   in 5 µl of template, with 8 replicate reactions for each dilution series. The lowest number

248   of nucleic acid targets detected in at least 95% of the 24 replicates was 50 DNA copies

249   (23 positive replicates). Therefore, the DL_{PCR} of SBV qPCR was determined as 50

250   genome copies per reaction.

251

252 3.3 Determination of the linearity range and the quantitation limit of the SBV qPCR assay

253 The linearity range and quantitation limit of the SBV qPCR assay was studied using three

254 independent 10-fold dilutions series of the 4.44 kb plasmid ranging from 10^8 to 10^2 DNA

255 copies in 5 µl of template. The results obtained for the three trials showed high PCR

256 efficiency, ranging from 91 to 93 %. The measured amount of SBV for each series and

257 each dilution level was compared to the theoretically expected amounts, to calculate the

258 individual absolute bias. Then, the mean bias, its standard deviation, and the linearity

259 uncertainty were determined. As shown in figure 3, at each dilution level, the absolute

260 bias value was less than the critical bias value (set at 0.25 $\log_{10}$) validating the linearity

261 over the entire calibration range. The combined linear uncertainty (U_{LIN}) of the SBV qPCR

262 was determined to be 0.12 $\log_{10}$ within the range from 2 $\log_{10}$ to 8 $\log_{10}$ SBV copies per

263 reaction. These results validate the calibration range and provide a quantitation limit of

264 100 SBV genome copies per reaction.

265

266 3.4 SBV quantitation in honey bee samples from symptomatic colonies

267 Figure 4 shows the mean SBV genomic loads obtained for SBV-symptomatic larvae,

268 asymptomatic larvae, pupae, and adult bees from symptomatic colonies in four French

269 apiaries. The SBV genomic loads were 2.9 × 10^7 to 2.1 × 10^{14} SBV copies per

270 symptomatic larvae, 7 × 10^4 to 3.3 × 10^{11} SBV copies per asymptomatic larvae, 2.3 × 10^4

271 to 1.1 × 10^{10} SBV copies per pupae and 2.2 × 10^3 to 3.4 × 10^{11} SBV copies per adult bee.

272 The mean of the SBV genomic load observed in SBV-symptomatic larvae was

273 significantly higher than the mean of the genomic load observed in the other samples

274 (p=0.004). No significant difference in SBV genomic load was observed between the

275 other sample types.

276

277 **4. Discussion**

278

10

279   Sacbrood virus is widely distributed throughout the world and is considered to be one of

280   the most common bee virus infections. Unlike most of the other bee viruses, sacbrood

281   virus may be reliably diagnosed by the distinctive and specific symptoms of infection in

282   larvae (Ball, 1996). Several previous works studied sacbrood virus outbreaks in different

283   countries (Allen and Ball, 1996, Grabensteiner et al., 2001, Nielsen et al., 2008). Most

284   commonly, the outbreaks of this disease occur in spring and early summer, when the

285   colony is growing rapidly and large numbers of susceptible larvae are available (Bailey

286   and Ball, 1991; Ball, 1999; Grabensteiner et al., 2001). Colonies rarely show diseased

287   larvae because the adult bees detect and remove most of them during the early stages of

288   infection (Bailey and Fernando, 1972). However, the incidence of symptomatic SBV

289   colonies appears to be increasing in Denmark (Nielsen et al., 2008) and in France

290   (unpublished data).

291   In order to improve an early SBV detection and confirm disease diagnosis, a two-step

292   real-time RT-PCR assay based on MGB TaqMan technology was developed to quantify

293   SBV genome in bee samples and to determine a viral load threshold in relation with the

294   overt disease. The assay was based on the XP U47-600 standard recently developed by

295   the French Standards Institute (AFNOR) and followed the method of the previously

296   described assay for CBPV detection and quantification (Blanchard et al., 2007; Blanchard

297   et al., 2012). The reliability and sensitivity of the qPCR assay were assessed by

298   determining several key performance parameters, such as analytical specificity, detection

299   limit (DL_{PCR}), quantitation limit (QL_{PCR}) and linearity range. The analytical specificity was

300   first established *in silico* by a Blast search within the public nucleic acid databases and

301   was experimentally confirmed by exclusivity tests against other bee viruses, such as

302   CBPV, ABPV, IAPV, BQCV and DWV and by inclusivity tests against a panel of samples

303   from various geographical regions (Austria, Denmark, France, Sweden and Uruguay).

304   A phylogenetic analysis was performed on positive SBV samples. This analysis was

305   based on the 429 bp structural protein obtained with primer pair SBV-1f and SBV-2r

306   described by Grabensteiner et al., (2001). Results are consistent with previous

307 phylogenetic analysis performed by Grabensteiner et al., (2001) and Kukielka and

308 Sánchez-Vizcaíno, (2009), updated recently by Choe et al., (2012) with South Korean

309 isolates. Three distinct genetic lineages of SBV are obtained.

310 Isolates from France, Germany, Austria, Sweden and Denmark group together to form

311 the continental European genotype. This first group has a minor subclade which includes

312 the Uruguayan and British isolates. The second group, which constitutes the Asian

313 genotype, is formed by the Chinese, Nepalese, and Indian isolates, whereas the South

314 Korean ones group together to form the third group.

315 All the new sequences obtained in this study (French, Austrian, Danish, Uruguayan and

316 Swedish isolates) fit well into this phylogenetic analysis.

317 Bee samples from Austria, Sweden, Norway and Uruguay revealed a moderate SBV

318 genomic load (10^4 to 10^{10} SBV copies per bee), whereas pupae and larvae samples from

319 Denmark and France showed a higher one (over 10^{11} copies per individual). One sample

320 from Sweden presenting the highest SBV genomic load (10^{10} SBV copies) was prepared

321 from adult bees from a colony that showed many SBV symptomatic drone larvae. The

322 French and Danish samples were collected during SBV outbreaks, related to the

323 increasing number of clinical cases described in these two countries. Hence, these

324 results confirm that the RT-qPCR SBV quantitation assay was able to detect genetically

325 diverse isolates. Although Asian or Korean isolates were not tested, the primers and

326 probe were designed in order to also match with Asian and Korean sequences.

327 The detection and quantitation limits of the RT-qPCR assay were evaluated from dilutions

328 of the plasmid obtained from SBV-1f and SBV-2r primers described by Grabensteiner et

329 al., (2001). These dilutions were also used to establish the standard curve. The detection

330 limit of the SBV qPCR was shown to be 50 genome copies for a template volume of 5 µl

331 of cDNA, corresponding to 4000 SBV genome copies per individual. Based on the linear

332 regression of the standard curves, PCR efficiency was proven to be high, ranging from 91

333 to 93%. The PCR performance reliability was determined from the bias and the linearity

334 uncertainty, obtained for each dilution level. All absolute bias values were less than the

335   critical bias value defined at 0.25 $\log_{10}$, validating qPCR linearity from 2 $\log_{10}$ to 8 $\log_{10}$

336   SBV copies (5 µl of cDNA). The quantitation limit was thus defined at 100 SBV genome

337   copies for a template volume of 5 µl of cDNA (i.e. 8000 copies / invividual), with a

338   measurement uncertainty evaluated at 0.12 $\log_{10}$. For diagnostic purposes, samples

339   containing fewer than 50 and 100 SBV genome copies per reaction were considered as

340   "SBV not detected" or "inferior to the QL_{PCR}" respectively.

341   The RT-qPCR SBV assay was then applied to a field survey carried out in France in

342   2012, where typical signs of SBV were observed in four apiaries followed by the "Brigade

343   Nationale d'Enquêtes Vétérinaires et Phytosanitaires" (BNEVP). The mean of SBV

344   genome copies obtained for larvae showing typical clinical symptoms ($10^{12.18 +/- 2.69}$ SBV

345   genome copies per individual) was significantly higher than the mean obtained for

346   asymptomatic larvae, pupae, and adult bees containing respectively $10^{7.05 +/- 1.96}$, $10^{6.98 +/-}$

347    $^{1.88}$ and $10^{6.6 +/- 2.79}$ SBV genome copies per individual. However, in some cases, the SBV

348   viral load obtained for asymptomatic larvae and adult bees reached 10^{11} SBV genome

349   copies/individual while the viral load observed in symptomatic larvae from the same

350   colony exceeded 10^{13} SBV genome copies/individual. This global correlation between the

351   SBV titres of symptomatic larvae and adult bees may be due to the hygienic behaviour of

352   adult bees which, when they detect and remove larvae during the early stages of infection

353   to reduce the risk of contamination, become infected by ingesting the virus-rich ecdysial

354   fluid of diseased larvae (Bailey and Fernando, 1972).

355   A similar survey was conducted in the same apiaries by the BNEVP in 2011, when few

356   clinical symptoms of SBV were observed. The SBV mean viral loads obtained for

357   asymptomatic larvae (72 samples) and pupae (81 samples) were $10^{5.56 +/- 1.27}$ and $10^{5.2 +/-}$

358    $^{1.69}$ SBV genome copies per individual respectively, while the SBV viral loads obtained for

359   symptomatic larvae and pupae (2 samples of each) exceeded 10^{13} and 10^{12} SBV genome

360   copies per individual respectively (data not shown). Furthermore, previous studies

361   showed that the SBV viral loads of pupae and adult bees from asymptomatic honey bee

362   colonies were evenly distributed at around 10^{9} SBV genome copies (Gauthier et al.,

363   2007). These results suggest that a lower threshold of SBV viral load correlated with

364   overt disease within a colony may be set at 10^{10} SBV genome copies per individual,

365   irrespectively of the bee sample (house bees, pupae and larvae). This threshold

366   corroborates results obtained with samples from Denmark (pupae and larvae with SBV

367   viral load exceeding 10^{11} and 10^{13} per individual respectively) where regular SBV

368   outbreaks have been described in recent years.

369   In conclusion, the RT-qPCR SBV assay developed in this study showed an excellent

370   performance according to the AFNOR XP U47-600 standard. Its application through field

371   surveys enabled the definition of a threshold of SBV viral load correlated with the overt

372   disease. Further investigations are in progress, in the first place to evaluate the entire

373   method as described by Blanchard et al., (2012) with purified SBV, and in the second

374   place to evaluate its specificity towards isolates from Asia and Korea where SBV

375   outbreaks were described (Grabensteiner et al., 2001; Choe et al., 2012)

376

385

386

387 **Captions to figures**

388

389 Table 1

390 Origin of the studied SBV isolates used for the phylogenetic analysis: isolate reference,

391 sample type, country of origin, sequence label and GenBank Accession number.

392

393 Figure 1

394 Representation of the SBV genome with the location of the 429 bp fragment

395 (Grabensteiner et al., 2001), the location of the 70 bp amplicon obtained with the SBV

396 qPCR assay described in this paper and the extremely high degree of sequence

397 conservation for this assay, across all SBV genotypes.

398

399 Figure 2

400 Maximum likehood phylogenetic tree of SBV sequences, based on a 429 bp segment of

401 the N-terminal part of the SBV polyprotein (nucleotides 241-669 according to reference

402 sequence AF092924). New sequences obtained in this study are in grey box (FRA2,

403 FRA3, FRA4, FRA5, AUT2, AUT3, URY1, SWE2, DNK1, DNK2). The number of each

404 node represents the bootstrap values as the result of 500 replicates. Bootstrap values <

405 50% were omitted. The scale corresponds to the number of substitution per site.

406

407 Figure 3

408 Performance of linear regression for the SBV qPCR. Mean bias (mb) was determined for

409 each load level, bars represent the linearity uncertainty (U_{LINi}) given by the formula: $2[\sqrt{}$

410 $SD^2 + (mean\ bias)^2]$ where SD is the standard deviation of the measured values.

411

412 Figure 4

413 Distribution of SBV viral load mean assessed by RT-qPCR assay in various categories of

414 samples from symptomatic hives: larvae with typical signs of SBV, larvae, pupae and

415   bees without clinical signs. The results are expressed as the mean SBV genome copies

416   per individual. Bars represent the standard deviations. (**significant difference at p < 0.01

417   within the various categories of samples).

418

419

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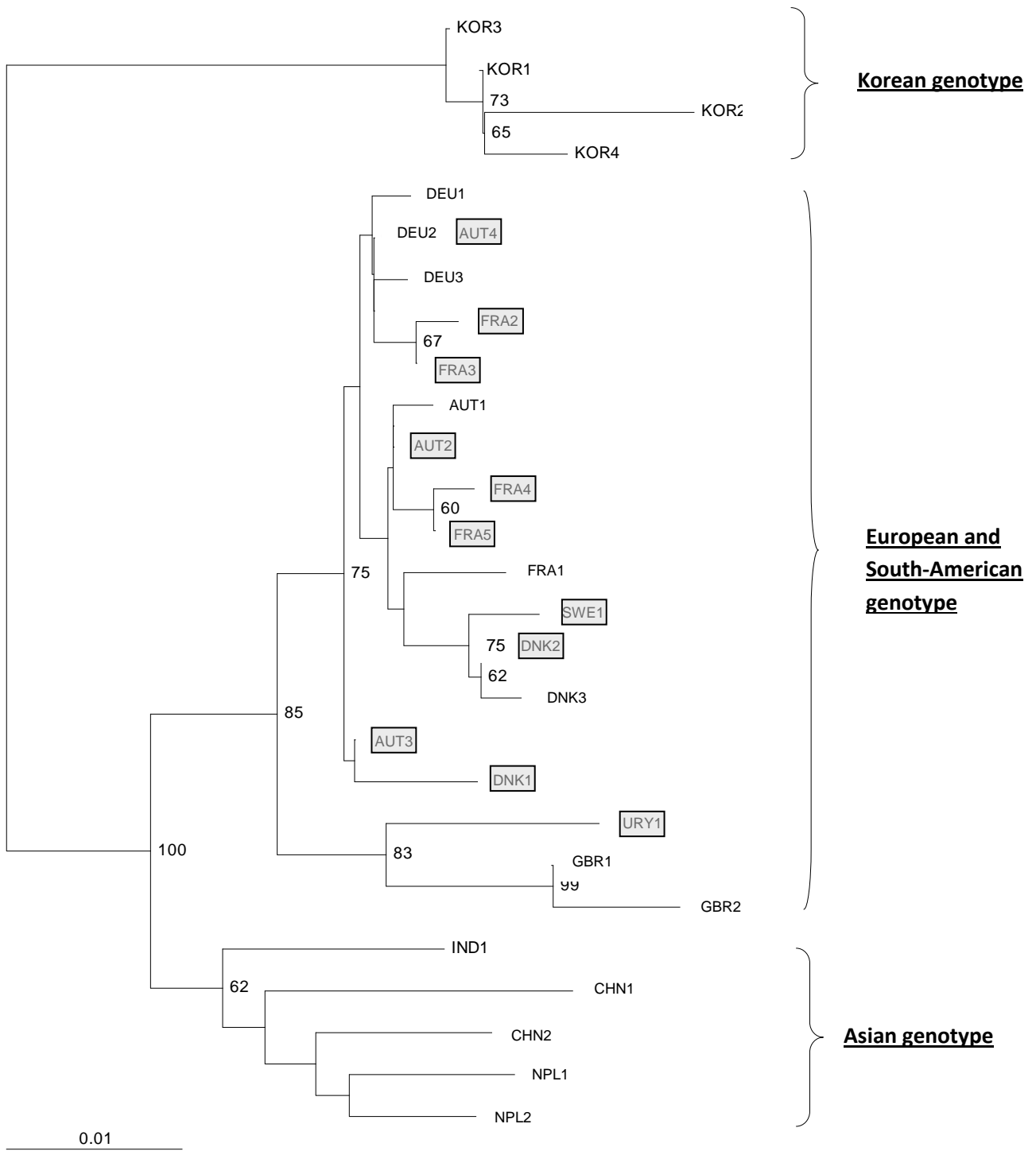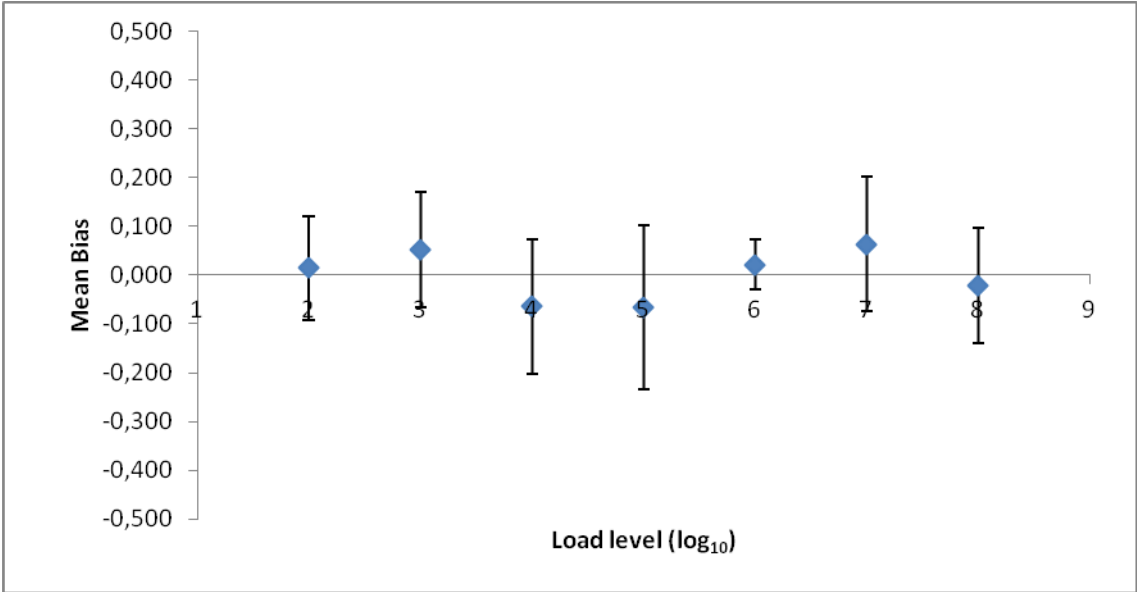512   chain reaction. J. Virol. Methods 179, 195-200.

Figure 1

SBV (~8800 nt)
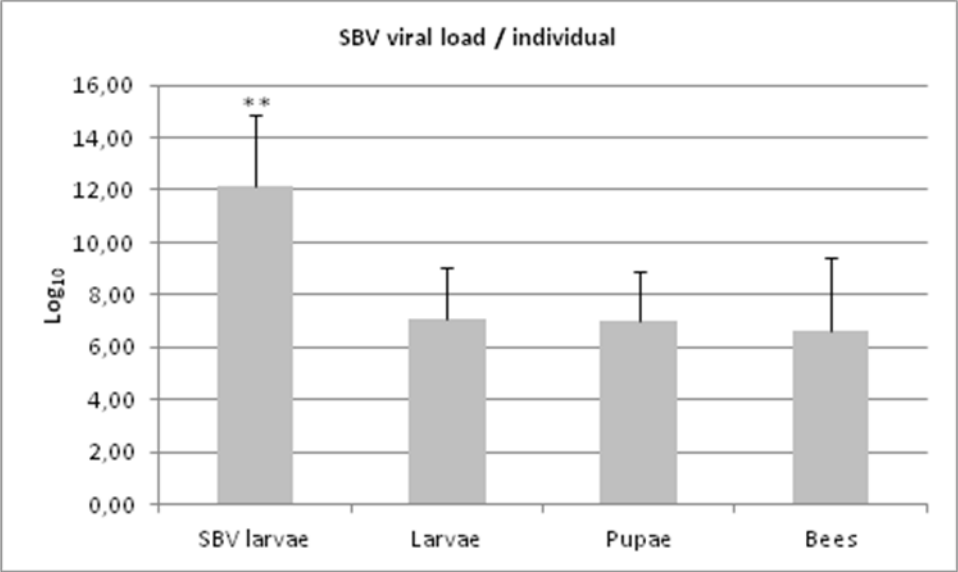
429 nt

70 nt

	SBV-F434	SBV-P460	SBV-R503

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                SBV-F434                      SBV-P460                      SBV-R503
         1231231231231231231231231231231 2 312312312312312312312312 3 1231 2 3123123123123123123123 1
SBV-GBR  AACGTCCACTACACCGAAATGTCCAG TGATGAGAGTGGACGAAGA ATCT GGAATGTTAGACGCGCAGTGT
SBV-URY
SBV-DNK
SBV-SWE
SBV-DEU
SBV-AUT
SBV-FRA
SBV-CHN  AACGTCCACTATGCCGAAATGTCCAC TGATGAGAGTGGACGAAGA ATCT GGAATGTTAGACGTGCAGTGT
SBV-NPL
SBV-IND
SBV-KOR  AATGTCCACTACACCGAAATGTCCAG TGATGAGAGTGGACGAAGA ATCT GGAATGTTAGACGTGCGGTGT
         **  *******  *********** **  ******************* **** ************  **  ****
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Figure 2

Figure 3

Figure 4

Table 1

Isolate[1]	Sample	Origin	Label	GenBank accession no.
D12-2024/S1859	Larvae	France	FRA 2	**KC513752**
D12-2024/S1861	Larvae	France	FRA 3	**KC513753**
20100203/24	Larvae	France	FRA 4	**KC513754**
20100020/1	Larvae	France	FRA 5	**KC513755**
155/1030/1253	Bees	Austria	AUT 2	**KC513758**
1006	Bees	Austria	AUT 3	**KC513759**
1003	Bees	Austria	AUT 4	**KC513760**
KA 2	cDNAs	Uruguay	URY 1	**KC513751**
JR6	cDNAs	Sweden	SWE 1	**KC513750**
BOX	cDNAs	Denmark	DNK 1	**KC513756**
12024	Larvae / Pupae	Denmark	DNK 2	**KC513757**
Rothamstead	Larvae	United Kingdom	GBR 1	**AF092924**
UK	Larvae	United Kingdom	GBR 2	**AF284616**
Austria	Larvae	Austria	AUT 1	**AF284617**
Germany 1	Larvae	Germany	DEU 1	**AF284618**
Germany 2	Larvae	Germany	DEU 2	**AF284619**
Germany 8	Larvae	Germany	DEU 3	**AF284625**
Sophia R80	Bees	France	FRA 1	**AY152712**
T73/05A	Bees	Denmark	DNK 3	**EF570887**
India	Larvae	India	IND 1	**AF284626**
Nepal 1	Larvae	Nepal	NPL 1	**AF284627**
Nepal 4	Larvae	Nepal	NPL 2	**AF284629**
China	Larvae	China	CHN 1	**AF469603**
CSBV / LN	Larvae	China	CHN 2	**HM237361**
Korean	Bees	Korea	KOR 1	**HQ322114**
4	Bees	Korea	KOR 2	**HQ916827**
11	Bees	Korea	KOR 3	**HQ916833**
13	Bees	Korea	KOR 4	**HQ916834**

[1] Isolate reference given by our colleagues