Development and validation of a real-time two-step RT-qPCR TaqMan® assay for quantitation of Sacbrood virus (SBV) and its 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*.

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;

Kukielka and Sánchez-Vizcaíno, 2009). Although real-time RT-PCR detection methods have been recently developed for the detection and the quantitation of SBV (Chantawannakul et al., 2006; Gauthier et al., 2007; Kukielka and Sánchez-Vizcaíno, 2009; Lock et al., 2012; Yoo et al., 2012; Evison et al., 2012), few data are available on the SBV viral titres of larvae samples presenting typical symptoms of SBV.

In this paper, a new real-time two-step RT-PCR based on the TaqMan® technology to quantify SBV was described. This SBV RT-qPCR was then validated according to the XP U47-600 French standard (as already described for chronic bee paralysis virus (CBPV) by Blanchard et al., (2012)). Finally, this method was assessed on field samples collected from an outbreak of sacbrood in France in 2012.

The development of this quantitative assay for SBV genomic RNA was performed using TaqMan® PCR with minor groove binder (MGB) probe technology. A DNA probe with a conjugated MGB group forms an extremely stable duplex with single-stranded DNA targets, allowing the use of shorter probes. The shorter probe length endows MGB probes with greater sensitivity and lower fluorescent background compared to non-MGB probes (Kutyavin et al., 2000). The SBV RT-qPCR assay was validated for (i) the analytical specificity, (ii) the PCR detection limit (DL_{PCR}), (iii) the PCR quantitation limit (QL_{PCR}) and (iv) the linearity and efficiency of the qPCR assay. The method was then applied to assess the SBV genomic load in different categories of samples (larvae with or without typical SBV clinical symptoms, pupae and adult bees) coming from apiaries suffering from sacbrood.

2. Materials and methods

2.1 Real-time quantitative RT-PCR method

The SBV method development was based on the TaqMan® two-step real-time quantitative RT-PCR CBPV assay developed previously by Blanchard et al., (2012) using the Applied Biosystems 7500 System (Applera). The SBV-specific primers and probe

were designed using the Primer Express 3.0 software (Applera). The choice of primers and probe was constrained by absolute sequence conservation across all the SBV sequences deposited in GenBank, including the Thai, Chinese and Korean variants (Figure 1). The chosen primer set amplifies a 70 bp fragment located in the N-terminal part of the polyprotein region of the SBV genome: nucleotides 434 to 503 according to the reference sequence (GenBank accession no. AF092924, Ghosh et al., 1999). The forward primer is SBV-F434: 5'- AACGTCCACTACACCGAAATGTC and the reverse primer is SBV-R503: 5'- ACACTGCGCGTCTAACATTCC. The TaqMan® probe is SBV-P460: 5'- TGATGAGAGTGGACGAAGA, labelled with the fluorescent reporter dye FAM (6-carboxyfluorescein) at the 5' end and with the non-fluorescent quencher (NFQ) with MGB at the 3' end.

The PCR is performed in duplicate in a MicroAmp optical 96-well reaction plate, containing 1X Taqman® Universal PCR Master Mix with uracil-N-glycosylase (UNG - Applied Biosystems), 320 nM of each primer (SBV-F434 and SBV-R503), 200 nM of the qSBV probe, 1X Exo IPC Mix and 1X Exo IPC DNA from IPC VIC™ Probe (Applied Biosystems). UNG reduces false-positive results by destroying contaminating, dUTP-incorporated PCR products from previous runs. The Exo-IPC reagents are a pre-optimized blend of an Exogenous Internal Positive Control (IPC) DNA template plus corresponding primers and VIC-based probe, designed to distinguish true-negative results from false-negative results due to PCR inhibition. The VIC-based signal of the IPC amplification is detected separately from the FAM-based signal of the SBV amplification. The reaction volume was completed with 5 µl of template, either 10^2 to 10^8 copies of the SBV plasmid DNA standard (described below) or random-hexamer primed cDNAs, obtained as previously described in Blanchard et al., (2007), giving a total volume of 25 µl. The thermal cycling conditions are 2 min at 50°C (active temperature for UNG degradation of contaminating PCR-products), 10 min at 95°C (activation of AmpliTaq Gold DNA Polymerase and inactivation of UNG), followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Results are expressed as the

mean of the two replicates of each reaction. A biological positive control is included in each run, obtained from homogenate of symptomatic larvae. Two negative controls are also included in each run: a blank RNA extraction negative control and a template-free qPCR negative control, in which the sample is replaced by purified water.

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2.2 Analytical specificity

Analytical specificity was assessed as described by Blanchard et al. (2012). The *in silico* analysis was performed by a Blast search on the genome database with the 70-bp sequence of the SBV amplicon. The experimental specificity was evaluated by exclusivity and inclusivity tests, testing the assay's ability not to amplify any RNA that is not SBV (exclusivity) and to positively detect all strains of SBV (inclusivity). The exclusivity test was performed on cDNAs obtained from bee samples infected with other bee viruses, such as ABPV, CBPV, IAPV, BQCV or DWV. The inclusivity test was carried out on several SBV isolates from various geographic regions (Austria, Denmark, France, Sweden and Uruguay), coming from apiaries with or without clinical symptoms and thus constituting a panel of samples that best represents SBV genetic diversity. The genetic diversity among these isolates was assessed by a phylogenetic analysis of the primer-free sequences of a 469 bp amplicon located in the structural protein region, encompassing the 70 bp fragment of the SBV assay, amplified by conventional PCR using primers SBV-1f and SBV-2r described by Grabensteiner et al. (2001). The sequences were aligned to homologous SBV sequences obtained from GenBank (Table 1) using CLUSTAL_X (Thompson et al., 1997). Phylogenetic trees were constructed using the neighbour-joining (NJ), maximum likehood (ML) and maximum parsimony (MP) methods as implemented by the PHYLOWIN program (Galtier et al., 1996). Statistical support for the branching pattern was provided by bootstrap analysis involving 500 replicates. Branches with less than 50% bootstrap were omitted. The phylogenetic trees were drawn using TreeView (Page, 1996).

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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 $U_{LIN} = \left|\sqrt{\Sigma U_{LINi}^2} / k\right|$ where k is the

number of dilution levels. The quantitation limit of the assay is then determined by the first level load of the calibration range.

To evaluate the performance of the entire method (RNA extraction to qPCR analysis), the positive control, obtained by homogenisation of symptomatic larvae, was followed by means of a control chart. In order to be accepted, the quantitative result had to be within the acceptability limits. These limits were defined by the mean of the first ten values ± twice the standard deviation.

2.4 Application of the SBV RT-qPCR method on bee samples from honey bee colonies with SBV clinical symptoms

In 2012, clinical symptoms of SBV were observed in French apiaries by the “Brigade Nationale d'Enquêtes Vétérinaires et Phytosanitaires” (BNEVP). Four apiaries located in three French departments (Loiret, Lot et Garonne and Vendée) were visited in April 2012. Three to five hives were sampled from each apiary. Samples of bee larvae showing typical SBV clinical symptoms were collected (12 samples of 2 to 5 larvae per hive), together with samples of asymptomatic larvae from the same hives (12 samples of 15 larvae per hive), samples of pupae (12 samples of 10 pupae per hive) and samples of in-house bees (12 samples of 10 bees per hive). Sample processing, RNA extraction and cDNA synthesis were performed as described in Blanchard et al. (2007). Five microliters of the cDNA were used as template for the qPCR.

2.5 Statistical analysis

The SBV genomic loads obtained from the bee samples were analysed by the Two-sample Kolmogorov-Smirnov and the Kruskal-Wallis non-parametric tests using the SYSTAT 9 computer software package (SPSS Inc.).

3. Results

3.1 Analytical specificity

In order to assess the specificity of the SBV assay, the chosen 70 bp sequence was searched *in silico* by a Blast analysis of the DNA public database. Only SBV sequences were identified by this search. Experimentally non significant amplification was detected in cDNAs obtained from ABPV, CBPV, IAPV, BQCV and DWV, which implies an exclusive specificity.

The inclusivity test was carried out on different isolates from Austria, France, Denmark, Sweden and Uruguay. A phylogenetic analysis was realised with isolates that gave a positive result in conventional PCR. SBV sequences from GenBank were included in this study. All the methods used for the inclusivity test (NJ, ML and MP) gave similar profiles. Results using the ML method are shown in figure 2. This analysis (realised on sequences of the SBV polyprotein region) revealed that all the European isolates (Austria, France, Denmark and Sweden) are included in a group close to the UK and Uruguay genotypes, whereas the Asian and Korean genotypes are included in an another group (Figure 2). Afterwards, samples of bees, larvae, pupae or cDNA were tested by SBV qPCR. The SBV genomic loads observed in adult bees and cDNAs were moderate, with 10^5 to 10^7 SBV copies per bee and 10^4 to 10^7 SBV copies per µl of cDNA. On the other hand, SBV genomic loads observed in larvae and pupae samples were much higher, over 10^{11} copies per individual.

3.2 Determination of the detection limit of the SBV qPCR assay

The detection limit of the SBV qPCR assay was determined using three independent 6-step, 2-fold dilutions series of the 4.44 kb plasmid ranging from 200 to 6.25 DNA copies in 5 µl of template, with 8 replicate reactions for each dilution series. The lowest number of nucleic acid targets detected in at least 95% of the 24 replicates was 50 DNA copies (23 positive replicates). Therefore, the DL_{PCR} of SBV qPCR was determined as 50 genome copies per reaction.

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.

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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

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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.,

2007). These results suggest that a lower threshold of SBV viral load correlated with overt disease within a colony may be set at 10^{10} SBV genome copies per individual, irrespectively of the bee sample (house bees, pupae and larvae). This threshold corroborates results obtained with samples from Denmark (pupae and larvae with SBV viral load exceeding 10^{11} and 10^{13} per individual respectively) where regular SBV outbreaks have been described in recent years.

In conclusion, the RT-qPCR SBV assay developed in this study showed an excellent performance according to the AFNOR XP U47-600 standard. Its application through field surveys enabled the definition of a threshold of SBV viral load correlated with the overt disease. Further investigations are in progress, in the first place to evaluate the entire method as described by Blanchard et al., (2012) with purified SBV, and in the second place to evaluate its specificity towards isolates from Asia and Korea where SBV outbreaks were described (Grabensteiner et al., 2001; Choe et al., 2012)

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Captions to figures

Table 1

Origin of the studied SBV isolates used for the phylogenetic analysis: isolate reference, sample type, country of origin, sequence label and GenBank Accession number.

Figure 1

Representation of the SBV genome with the location of the 429 bp fragment (Grabensteiner et al., 2001), the location of the 70 bp amplicon obtained with the SBV qPCR assay described in this paper and the extremely high degree of sequence conservation for this assay, across all SBV genotypes.

Figure 2

Maximum likehood phylogenetic tree of SBV sequences, based on a 429 bp segment of the N-terminal part of the SBV polyprotein (nucleotides 241-669 according to reference sequence AF092924). New sequences obtained in this study are in grey box (FRA2, FRA3, FRA4, FRA5, AUT2, AUT3, URY1, SWE2, DNK1, DNK2). The number of each node represents the bootstrap values as the result of 500 replicates. Bootstrap values < 50% were omitted. The scale corresponds to the number of substitution per site.

Figure 3

Performance of linear regression for the SBV qPCR. Mean bias (mb) was determined for each load level, bars represent the linearity uncertainty (U_{LINi}) given by the formula: $2[\sqrt{SD^2 + (\text{mean bias})^2}]$ where SD is the standard deviation of the measured values.

Figure 4

Distribution of SBV viral load mean assessed by RT-qPCR assay in various categories of 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).
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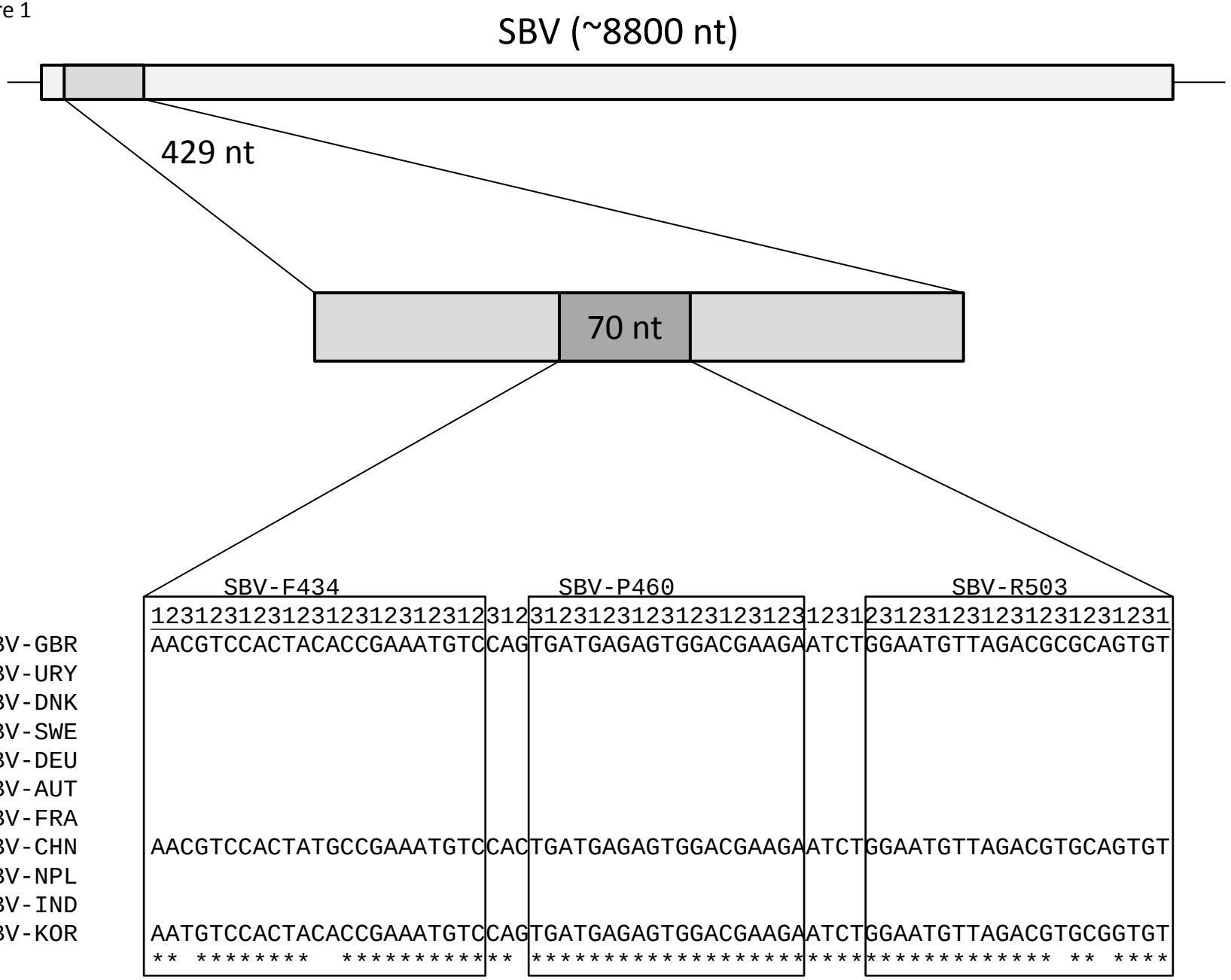
Figure 1


SBV (~8800 nt)
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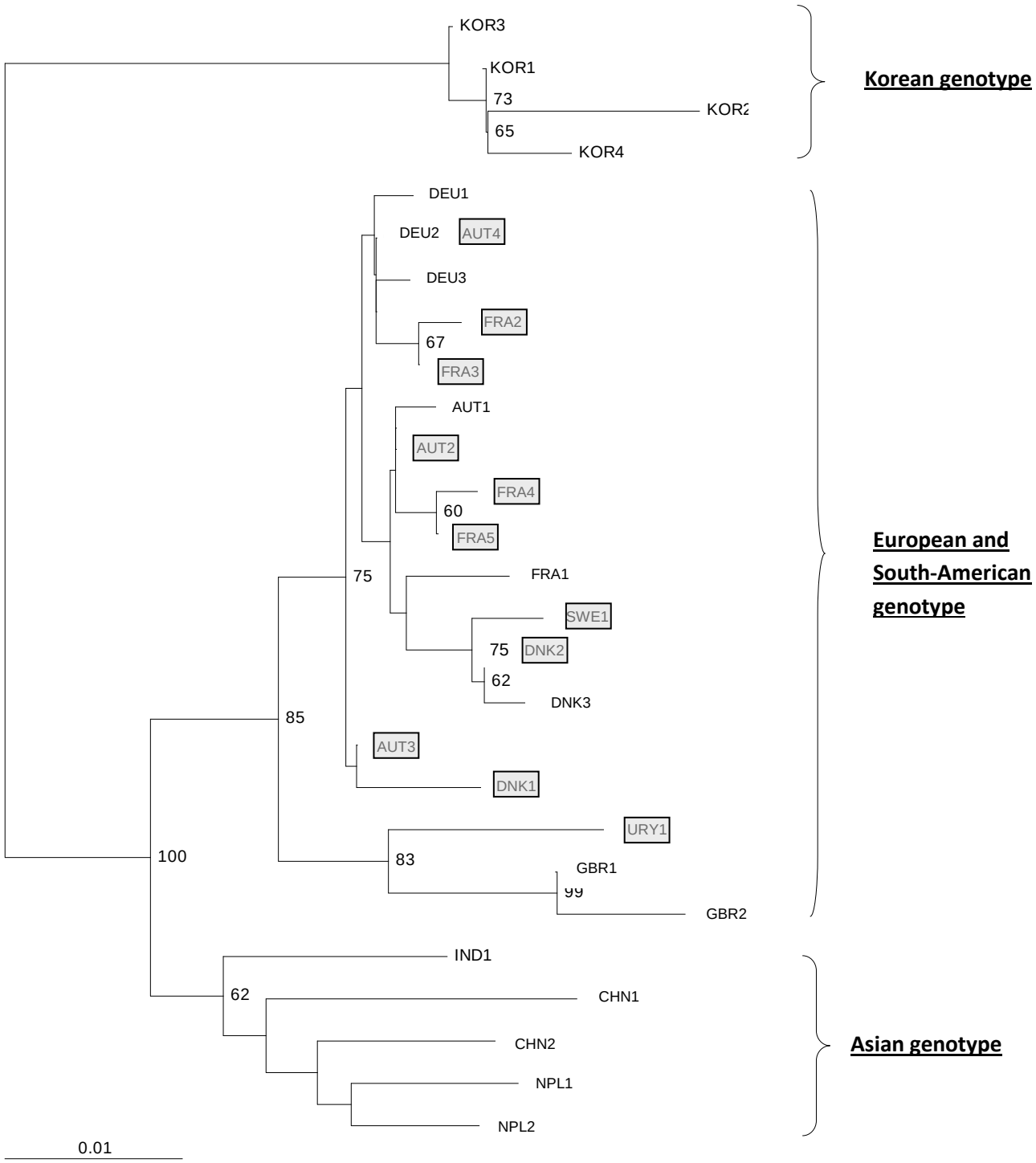
70 nt
SBV-F434
SBV-P460
SBV-R503
SBV-GBR AACGTCCACTACACCGAAATGTCCAGTGATGAGAGTGGACGAAGAATCTGGAATGTTAGACGCGCAGTGT
SBV-URY
SBV-DNK
SBV-SWE
SBV-DEU
SBV-AUT
SBV-FRA
SBV-CHN AACGTCCACTATGCCGAAATGTCCACTGATGAGAGTGGACGAAGAATCTGGAATGTTAGACGTGCAGTGT
SBV-NPL
SBV-IND
SBV-KOR AATGTCCACTACACCGAAATGTCCAGTGATGAGAGTGGACGAAGAATCTGGAATGTTAGACGTGCGGTGT

Figure 2


KOR3
KOR1
73
KOR2
65
KOR4
Korean genotype
DEU1
DEU2
AUT4
DEU3
FRA2
67
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AUT1
AUT2
FRA4
60
FRA5
75
FRA1
SWE1
75
DNK2
62
DNK3
85
AUT3
DNK1
URY1
100
83
GBR1
99
GBR2
European and South-American genotype
IND1
62
CHN1
CHN2
NPL1
NPL2
Asian genotype
0.01

Figure 3

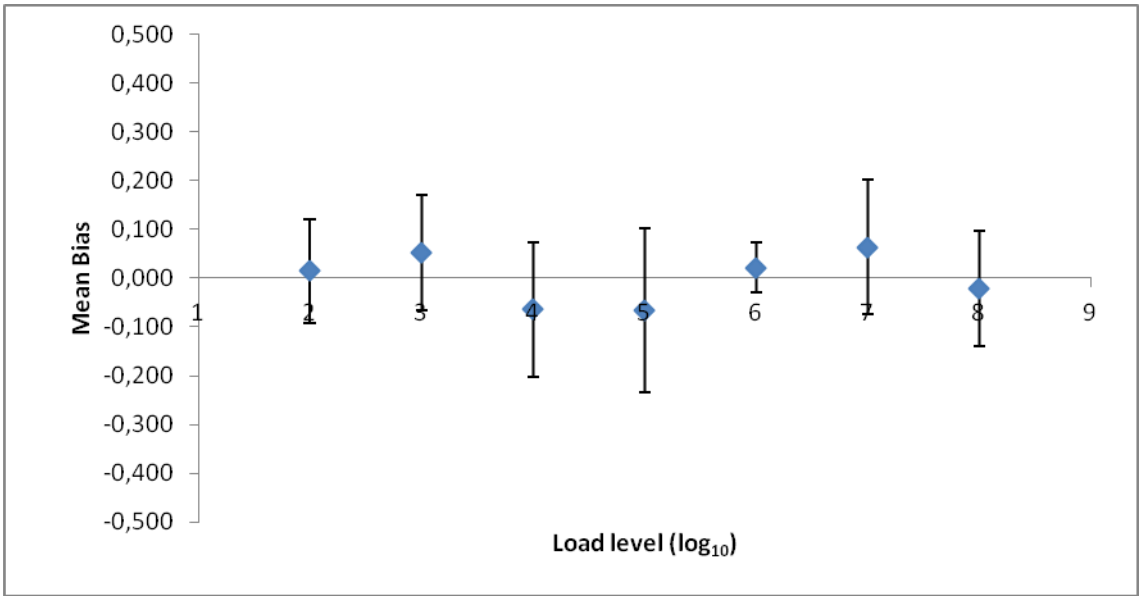
0,500
0,400
0,300
0,200
0,100
0,000
-0,100
-0,200
-0,300
-0,400
-0,500
Mean Bias
1
2
3
4
5
6
7
8
9
Load level (log10)

Figure 4

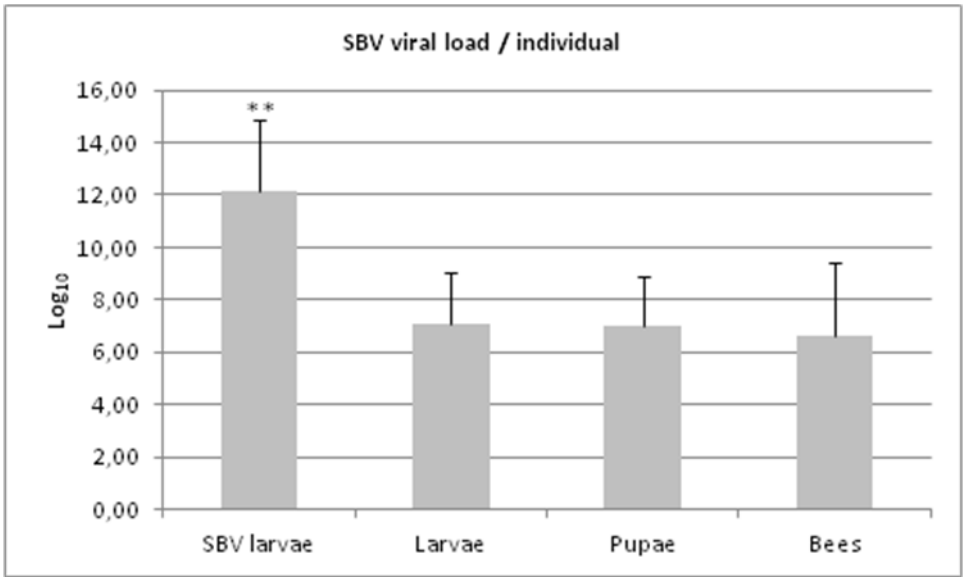
SBV viral load / individual
16,00
14,00
12,00
10,00
8,00
6,00
4,00
2,00
0,00
Log10
**
SBV larvae
Larvae
Pupae
Bees

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