

A severe sacbrood virus outbreak in a honeybee (*Apis mellifera* L.) colony: a case report

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ABSTRACT: A honeybee colony, part of an apiary of nine, showed abnormalities in brood pattern and was thus presented for study. A classic veterinary medicine approach has allowed the diagnosis of a severe case of sacbrood virus (SBV) confirmed by a high viral load in affected larvae. SBV is known to infect larvae of the honeybee (*Apis mellifera*), resulting in failure to pupate and ultimately death of infected larvae. Several contributing factors combined, among them the parasite *Varroa destructor*, have been identified in this particular affected colony to explain the clinical outbreak of the disease whereas, in the majority of cases, infected colonies remain asymptomatic. As no specific cure of honeybee viruses is available, the management of these contributing factors is essential, including feeding of colonies and control of the *Varroa* parasite. After implementation of management solutions, the colony rapidly recovered in six weeks, but did not recommence honey production and remained at higher risk of a winter collapse. An earlier control management would have been more effective: regular visits of the colonies by the beekeepers should be the rule in order to detect abnormalities and also to detect and eliminate as early as possible the combination of factors that contribute to the proliferation of the virus.

Keywords: *Apis mellifera*; contributing factors; synergism; sacbrood virus; *Varroa destructor*

The sacbrood virus was the first honey bee virus to be studied and one of the first to be identified (White 1917; Bailey et al. 1964). White (1917) gave a complete description of the symptoms of SBV, particularly at the different stages of the larvae. He pointed out that it was readily distinguished from other brood diseases such as European foulbrood. Sacbrood virus is common in colonies and is widely present in most locations. In French apiaries, the prevalence of the virus ranges as follows: SBV is present in approximately 80% of pupae and in approximately 85–86% of adult bees (Tentcheva et al. 2004; Mouret et al. 2013). A seasonal variation of viral infections has been demonstrated: SBV is prominently found in spring and summer in both adults and pupae (Tentcheva et al. 2004). No data are available regarding the prevalence of the SBV disease in France but the majority of infections probably remain asymptomatic given the very large spread of the virus. In France and elsewhere, some authors have reported overt infection in brood or SBV detection in colonies without clinical symptoms

(Grabensteiner et al. 2001; Tentcheva et al. 2004; Antunez et al. 2006; Nielsen et al. 2008; Blanchard et al. 2014). Larvae infected by this Iflaviridae (King et al. 2011) fail to pupate four days after they have been sealed in their brood-comb cells by the worker bees (White 1917; Bailey et al. 1964). Then, an ecdysial fluid rich in virus accumulates beneath their unshed skin, forming the sac which gives its name to the disease (Bailey et al. 1964). Although SBV affects the brood, it can be isolated in adult bees which contribute to the spread of the virus. Adult bees can transmit the virus during exchanges (trophallaxis) and tasks (for example cleaning contaminated cells). As no clinical description of such a severe case of SBV is available, we here report a complete case including a contributing factor analysis.

Case description

In the middle of July 2014, a beekeeper contacted our veterinary clinic because, during a routine visit,

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he noticed an irregular brood pattern in one of his colonies. This apiary is composed of nine hives. It is located in a mountain region in the centre of France (N45°10.126'/E 002°43.371', altitude 1120 metres). The beekeeping farm features are: one single apiary of nine colonies, Dadant hives with ten frames (common wooden beehives), the local black honeybee *Apis mellifera mellifera* is the honeybee strain reared. The aforementioned features of this farm are usual in French apiculture. The anamnesis given by the beekeeper was complete, which is quite rare, and the beekeeping husbandry in question can be considered as following Good Beekeeping Practices (*Varroa* control, colony and material management, etc.). The origin of the affected colony was a nucleus made on the 1st of April 2014 with three frames of capped and uncapped brood, one frame with pollen and honey and adult bees removed from a colony considered to be strong and in good health. The emergence of a young queen occurred on the 12th of April 2014. According to the beekeeper, the “good quality” of this queen (morphology, health, fertilisation) was controlled on the 15th of May 2014 and the colony was estimated to be strong enough in July to start honey production.

On the 12th of July 2014, a veterinary practitioner performed a clinical examination of the whole apiary, beginning with the supposed healthy colonies. During the examination of the eight non-symptomatic colonies, no clinical signs of infection were noted (strength, brood, adult bees, pollen and honey stores appeared normal). Checking for *Varroa destructor* by uncapping drone cells revealed a mi-

nor presence of the parasite (less than 10% of cells infested). The clinical examination of the affected colony first showed an apparently normal activity on the alighting-board with no abnormalities in front of the hive (no cadavers or affected adult bees). When opening the hive, the colony appeared usually strong with all the inter-frames spaces occupied and the wax frames regularly covered with bees. The population was estimated at approximately 28×10^3 bees according to the Liebfeld method (Imdorf et al. 2010). Very few drones were present within the bee hive and no drone brood was reared by bees. In adult bees, no clinical, morphological or behavioural symptoms were noted (trembling, crawling, flight difficulties, aggressiveness, deformed wings, abnormal colour, diarrhoea, constipation, etc.). However, pollen and honey stores were relatively poor compared with the other colonies from the same apiary and in relationship to the population size. The brood pattern appeared irregular (scattered), smell-free with punctures on seal brood (Figure 1). An abnormally high quantity of dead larvae was present in the cells. Among the six brood combs, five were severely affected with approximately 80% of brood area affected. Affected cells were either capped or recently uncapped by cleaner bees (Figure 2). Some punctures, variable in size and in number, were frequently noticed. Dead larvae were coloured from slightly yellow to dark brown. Affected un-adherent larvae were easily removed from the cell. When removed, most of them had the appearance of a distended sac containing a fluid within a thin cuticle wall (Figure 3). This accu-

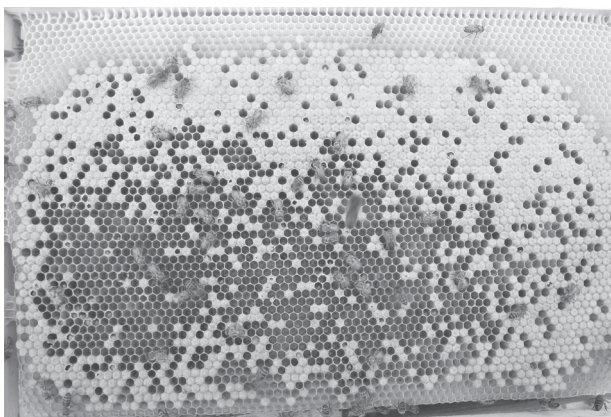


Figure 1. Aspect of the irregular brood (especially in the lower left quarter) of the colony affected by sacbrood virus and *Varroa* parasitism

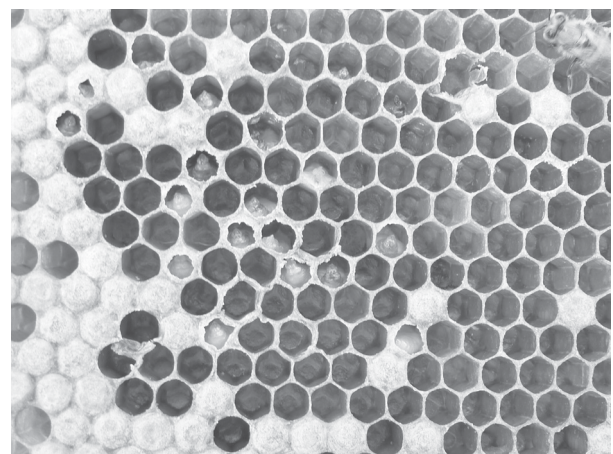


Figure 2. Zoom in an affected brood area with recently uncapped cells containing dead larvae. Larvae infested by SBV are easily removed (unadherent) and appeared slightly yellow with a dark head

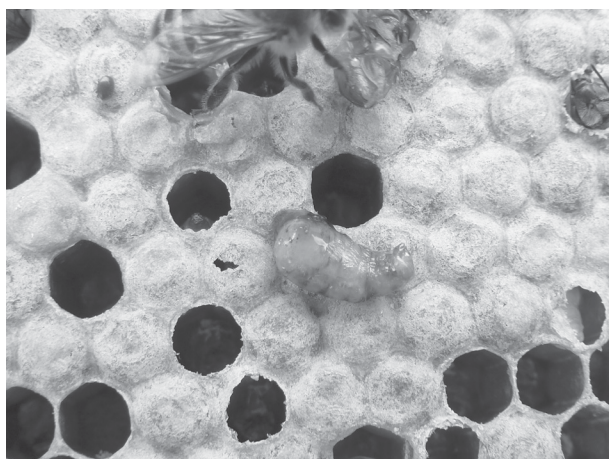


Figure 3. Dead larvae, once removed from affected cells, look like a distended sac containing a fluid with a thin cuticle wall. Note the mite on the top left corner

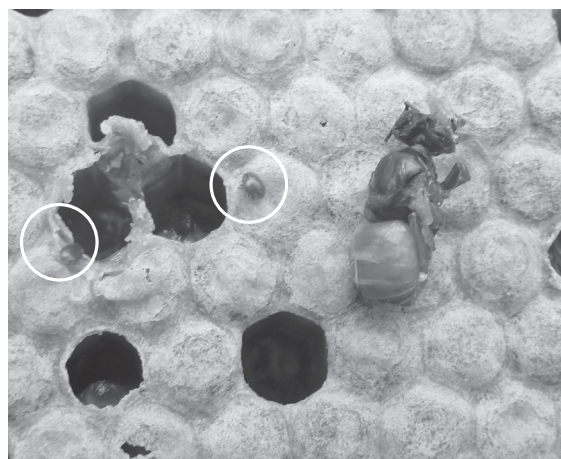


Figure 4. An infested cell, uncapped during the clinical examination, containing a dead nymph and two mites (white circles)

mulated liquid consisted of a more or less granular-appearing mass suspended in a watery fluid. Some capped worker-brood cells ($n = 25$) with normal appearance were uncapped to determine the presence of the *varroa* mite: 16% ($n = 4$) were infested by the mite (Figure 4), a percentage significantly higher than that found in the non-symptomatic colonies of the same apiary (with the same applied method, the presence of *Varroa destructor* parasitism in the eight other colonies ranged from 0% to 4%).

Hypothesis of diagnosis

The clinical exam highlighted the main factors affecting the brood: a moderate *Varroa* mite infestation rate and a great number of dead larvae, these dead larvae forming a sac containing an ecdysial fluid. The symptoms observed and the characteristics of the affected larvae can easily be associated with sacbrood virus (SBV) infection. Differential diagnosis must be considered with diseases of the brood such as European and American foulbrood diseases (bacterial diseases) and Chalkbrood disease (mycosis). However, the symptoms of those diseases differ sufficiently from the symptoms presented by this colony that neither could be retained as the first hypothesis. To confirm the diagnosis of sacbrood virus, some infected larvae were sampled and preserved by negative freezing (-18°C) before laboratory analysis. A sample was ice-shipped to the Montpellier SupAgro laboratory for further molecular experiments.

Laboratory analysis

The frozen sample (a pool of eight honeybee larvae residues) was crushed at 4°C in a cold mortar and homogenised in TN extraction buffer (10mM Tris–400 mM NaCl buffer, pH 7.5). The sample was centrifuged for 1.5 min at $1000 \times g$ at 4°C to separate supernatant from sand. Total RNA was extracted from an aliquot of supernatant with the NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer's recommendations. A DNase treatment was performed. Total RNA was resuspended in RNase-free water and quantified using spectrophotometry. Next, cDNAs were retrotranscribed from 3 μg of total RNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen), utilising random hexamers and oligo dT, according to the manufacturer's recommendations. An absolute quantification of sacbrood bBee virus (SBV) – i.e., an estimation of the number of copies contained in the sample – was performed using quantitative PCR (primer sequences, Table 1). A non-template control (H_2O) was included in each reaction run. A house-keeping gene, *A. mellifera*-actin, (primer sequences, Table 1) was used to ensure the nucleic acid extraction efficiency and quality. The primers (Eurogentec, Belgium) were used at a final concentration of $0.2\mu\text{M}$. All qPCR assays were performed with Brilliant II SYBR Green QPCR Low ROX Master Mix (Agilent Technologies) according to the supplier's recommendations on 5 μl of nucleic acid (cDNA) diluted in water (6 ng/ μl), in a 25 μl final volume. Reactions were run using the Mx3005P QPCR System (Stratagene)

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Table 1. PCR primer sequences

Primer name	5'-3' sequence	Primer size (bp)	Amplicon size (bp)	References
For-Actin1	AGGAATGGAAGCTTGCGGTA	20	181	Chen et al. 2005
Rev-Actin1	AATTTTCATGGTGGATGGTGC	21	–	
For-SBV	CGAGTGTGTGTGTGTAAAGAGA	23	340	Kukielka and Sanchez-Vizcaino 2009
Rev-SBV	CGAAGGGTGAAGTGTAGCAG	20	–	

apparatus under the following conditions: 10 min at 95 °C, and 40 amplification cycles (30 s at 95 °C, 1 min at 60 °C, and 30 s at 72 °C). An additional dissociation curve program (from 55 °C to 95 °C) allowed the discrimination of specific from non-specific amplicons. Each sample was run in duplicate. Run PCR data were analysed using the MxPro™ QPCR software. To perform an absolute quantitation, a standard curve was generated with ten-fold dilutions (10^9 to 10^1 copies) of purified PCR amplicons as template (Gauthier et al. 2007). Moreover a sample of ten young worker bees was carried out to verify the honeybee race using a morphological geometric analysis based on measures of wing venation (National Natural History Museum of Paris, Apiclass™ Software).

The morphological data confirmed the identification of the European Black honeybee *Apis mellifera mellifera*. The molecular analysis showed a viral load of 5.68×10^{11} SBV genome copies per larvae, confirming the suspicion of SBV.

Management

As no veterinary drugs are indicated for the care of honeybee virus infections, only management practices could be applied. We also suggested that the beekeeper feed his colony (Syrup 50/50 and frozen pollen every three days for three weeks), that he reduce the entrance of the hive, so as to limit spoliation by neighbouring colonies, and that he take action against the spread of *Varroa destructor* mite infestation. The whole apiary was kept under close surveillance and was regularly visited during the summer and autumn of 2014. The eight healthy colonies remained without clinical signs throughout this period and their honey production was high for this mountain region (from 12 to 19 kg). In contrast, in the diseased colony honey production remained non-existent. At the end of August, its population size became much lower with only four frames covered by bees (12×10^3 bees estimated using

the Liebefeld method) and the four lateral frames were entirely empty of bees, reserve or brood. In accordance with good beekeeping practices *Varroa destructor* chemical control was performed in the whole apiary after harvesting (on the 1st of August) with Amitraze (Apivar™, Veto-pharma laboratory, France) applied over the course of ten weeks. Lesions of SBV were reduced at six weeks and the brood appeared normal in September. The colony was then fed with sugar to facilitate winter survival and to compensate for a lack of honey stores.

DISCUSSION AND CONCLUSIONS

This clinical report is the first in which SBV and *Varroa destructor* have been clearly identified together and with such severe clinical signs affecting the brood (80% of the brood area affected). A careful clinical examination with a classic veterinary diagnosis approach and some common additional examination allowed confirmation of the suspected diagnosis by a quantification of virus load.

Blanchard and colleagues (2014) recently proposed a threshold of 10^{10} SBV genome copies per larvae, below which, a colony would appear healthy and remain without clinical signs of disease. This corroborates the results we obtain for samples exceeding 10^{11} genome copies per larvae. We did not, however, detect any clinical signs of SBV in the adult bees tested, although experimental effects of SBV have been described as follows: (i) an accelerated progression from young bees tending to become earlier foragers (Bailey and Ball 1991), (ii) the secretion of royal jelly is impaired in infected nurses compared to non-infected nurses (Du and Zhang 1985), (iii) and a strong aversion to eating or collecting pollen (Bailey and Fernando 1972). Also, the stock of pollen and honey stored in the affected beehive was significantly lower than that of other comparable colonies from the same apiary. This could be explained by a population drop

because of the higher mortality rate in larvae or, in accordance with experimental data, to a lower foraging effort. However local meteorological conditions had been unusually difficult for bees during the late spring and the early summer: whereas June was hot and dry, July was particularly wet and cold (source: www.meteofrance.fr). Consequently pollen resources were less abundant and less accessible for colonies. These challenging and adverse environmental conditions may have disrupted the development of the colony and affected fundamental elements in the lives of the bees causing problems such as protein and/or immune-deficiency (Alaux et al. 2010; Brodschneider and Crailsheim 2010), making bees more vulnerable to disease. The origin of the colony (artificial swarm) can be another risk factor because of a possible population unbalance (bad brood/bees rate). This could have affected the brood rearing. Finally, several contributing factors occurred simultaneously and a possible synergism allowed the clinical expression of the disease: a possible population unbalance, a marked lack of stores (especially of pollen) and confinement caused by the bad July weather. The genetics of the colony, in particular its hygienic behaviour, can contribute to the spread of the disease, but the colony became free of SBV lesions quickly despite the numerous dead larvae. The role of the parasite *Varroa destructor* in this outbreak is probably important even if the infestation rate seems to be moderate. Its effects on bees and broods are known and it is a factor in colony weakening (Le Conte et al. 2010). In addition, the mite could have played a significant role in the spread of SBV within the colony: (i) the virus has already been detected in mites and their saliva (Shen et al. 2005) and in a French study 45% of mites were found to be infected by SBV (Tentcheva et al. 2004); (ii) the ability of the mite to transmit the virus from infected to healthy pupae has been experimentally demonstrated (Ribiere et al. 2008). When infected, the parasite can also participate in the transmission of the disease, even though there is no actual proof of the replication of the virus in *Varroa destructor*. The fact that the mite infestation was shown to be greater in the affected colony compared to in the healthy ones is in line with the scientific evidence and conclusions of these related studies. Consequently, *Varroa* control indirectly contributes to the disease management and must be carried out. Genetic factors are sometimes considered as a potential contributing factor to SBV: according to Bailey (1967),

some strains of bees differ in their susceptibility to sacbrood virus and he encouraged the replacement of the queen by a new young local one (requeening), especially when the queen from the affected colony is imported. In our case, the queen was young (three months) and reared *in situ* by the beekeeper. To our knowledge, the European Black Honey bee used here is not known to be more sensitive to the SBV Virus than other strains. Nevertheless, changing the queen could be of benefit to the hygienic behaviour of the colony or could limit the vertical transmission of the disease (Chen et al. 2006). Moreover, genetic variations of the 'European genotype' of SBV have been reported suggesting distinct strains of the virus (Grabensteiner et al. 2001). However, little information is available concerning the virulence of these different viral strains.

At present, SBV disease treatment does not exist because of the lack of therapeutic means. An adapted management of contributing factors is the only way to improve the health of affected colonies. In our case, the better climatic conditions in August along with the efficient feeding of the bees and control of *Varroa* infestation probably helped the colony to recover its health status after six weeks. Concerning the colony survival during the next months, prognosis is usually optimistic with SBV. Nevertheless, winter collapse can occur due to an insufficient population size and relapse is possible in cases where the viral load is persistent.

The colony clinically affected by SBV was the only one in the apiary simultaneously affected by several contributing factors, which is the probable reason why an overt infection was able to develop. Even though the colony survived and recovered quickly a healthy brood, the population size severely decreased and it ceased to produce honey. A very early detection of the disease could have allowed the number of affected cells to be limited and might have contributed to maintaining productivity. Good beekeeping practices require frequent visits to all colonies at regular intervals (within the hive) by the beekeeper to check their health status. Such frequent visits will allow professionals (beekeepers and veterinarians) the opportunity to commence the management of contributing factors earlier in order to facilitate a more efficient and rapid recovery of the health of the colony and to limit the eventual spread of the disease. Thus, we consider that a minimum frequency of one visit per month is necessary, especially during the brood development.

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