

Prevalence of 12 infectious agents in field colonies of 18 apiaries in western France

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SUMMARY

A one-year survey of twelve infectious agents identified in honey bees (*Paenibacillus larvae*, *Melissococcus plutonius*, *Nosema apis*, *Nosema ceranae*, Acute Bee Paralysis Virus, Black Queen Cell Virus, Chronic Bee Paralysis Virus, Deformed Wing Virus, Israeli Acute Paralysis Virus, Kashmir Bee Virus, Sacbrood Bee Virus and *Varroa destructor* Virus 1) was performed in western France in 2009. During inspection, adult bee samples were collected four times a year from five colonies, in 18 apiaries. Sample contents were described and quantified by quantitative PCR methods. A high prevalence of the infectious agents studied was found both in terms of colonies and of apiaries, as well as very frequent co-infections within the same colony/apiary. These findings indicate a frequent infection of the apiaries and the colonies by most of the agents, and support the involvement of other weakening stressors in the disease outbreak.

Keywords: *Apis mellifera*, infectious agents, prevalence, RT-qPCR

Abbreviation: IA: Infectious Agent(s)

RÉSUMÉ

Prévalence de 12 agents infectieux dans 18 ruchers de l'ouest de la France

Un suivi de 12 agents infectieux connus chez l'Abeille domestique (*Paenibacillus larvae*, *Melissococcus plutonius*, *Nosema apis*, *Nosema ceranae*, Acute Bee Paralysis Virus, Black Queen Cell Virus, Chronic Bee Paralysis Virus, Deformed Wing Virus, Israeli Acute Paralysis Virus, Kashmir Bee Virus, Sacbrood Bee Virus and *Varroa destructor* Virus 1) a été réalisé durant l'année 2009 en France. Lors de l'inspection de la colonie, des prélèvements d'abeilles ont été effectués à quatre reprises durant l'année sur 5 colonies dans 18 ruchers. Une analyse moléculaire qualitative et quantitative (qPCR) a été réalisée. Une prévalence élevée des agents infectieux a été mise en évidence, tant à l'échelle des colonies qu'à l'échelle des ruchers. Ces résultats indiquent une infection importante par la plupart des agents infectieux et corrobore la thèse de l'intervention de nombreux agents stressants dans la déclaration d'une maladie apiaire.

Mots-clés : *Apis mellifera*, agents infectieux, prévalence, RT-qPCR

INTRODUCTION

Among pollinating animals, insects, including domestic bees (mainly *Apis mellifera*) play a major role {15}, {26}, {21}. In addition to their role in maintaining flowering plant biodiversity, bees are the highest economic value pollinators in intensive crop production all over the world, and contribute greatly to the efficiency of agricultural production {10}, {15}, {21}, {26}.

For several decades, a drastic decrease of the pollinating insect population has been reported in many countries by professionals and scientists with various disorders of the bee described {37}. The increase of this decline is a major concern from both biological and economic points of view and is the topic of a heated debate, mostly in the media, making it difficult for a rational, objective and scientific approach to this important issue. Currently, many factors are suspected to be involved in the evolution of colonies, including infectious agents such as viruses, bacteria, fungi and parasites {9}, {11}, {23}, {25}, {28}, {31}, {37}. Indeed, a

wide variety of microorganisms is associated with bees {2}, {17}. Like in other living organisms, asymptomatic carriage of infectious agents may be a common feature of honey bees {16} until risk factors are added and trigger the disease, but there is still a lack of field data to reinforce this hypothesis.

However, it is crucial to know the carriage of these infectious agents (IA) in field colonies, in order to modulate the consequences of a positive diagnosis of the presence of one or more of these IA. Precise knowledge of IA prevalence is also necessary to understand the phenomena now observed and to promote an accurate clinical approach to explain the role of the different stressors in the triggering and the development of bee colony collapse.

The purposes of this study were to assess 1) the prevalence of each IA in apiaries and in colonies, 2) the main co-infections occurring in colonies and 3) the survival of these colonies after the wintering season.

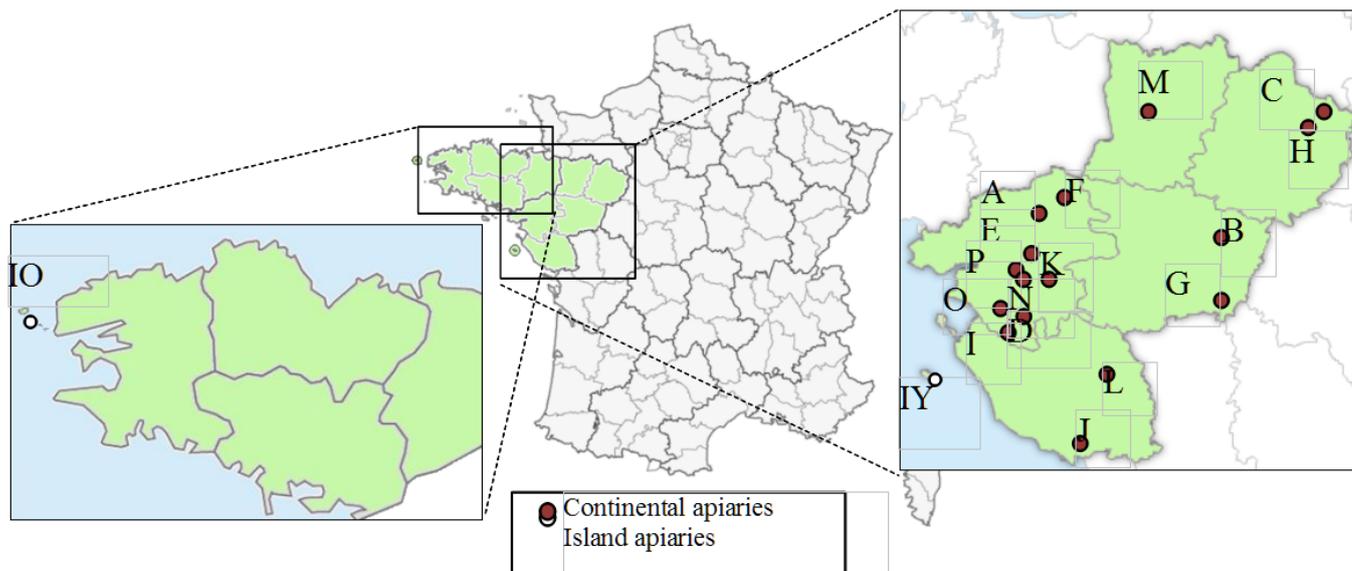


FIGURE 1: Location of the 16 continental and 2 island apiaries

MATERIALS AND METHODS

BIOLOGICAL MATERIALS: BEE SAMPLING.

A cohort of 18 beekeepers participated in this survey (Fig. 1): 16 continental apiaries from Pays de la Loire and two island apiaries, one located on the *V. destructor*-free Island of Ouessant (Ushant) (Brittany region) (apiary IO) and one on the Island of Yeu (Pays de la Loire region) (apiary IY).

Five colonies were randomly identified in each apiary. For each colony, samples of adult bees (30 to 50 individuals) were collected from brood frames at four periods during 2009: period 1 (end of April or beginning of May), corresponding to the start of honey foraging, period 2 (end of June or beginning of July), corresponding to the middle/end of honey foraging, period 3 (end of July or beginning of August) and period 4 (end of September or beginning of October) corresponding to preparation for wintering. Their evolution (live/dead) after wintering in March 2010 was also observed. Once collected, the samples were immediately frozen at -18°C and sent by refrigerated shipping to the Laboratoire de Pathovigilance et de Développement Apicole de Montpellier SupAgro-USAE for IA identification and quantification.

The analytic methods performed in the laboratory were carried out on the model developed by Gauthier et al. {16} and on the basis of genetic data mentioned by various authors {4}, {8}, {9}, {16}, {18}, {19}, {22}, {30}, {32}.

DATA AND STATISTICAL ANALYSIS.

The raw data obtained were Ct (threshold cycle) values, allowing to deduce IA loads (number of sequence copies

detected per bee) from standard curves. These results were calculated for each IA in each colony at each sampling period.

A total of 360 (18 apiaries x 5 colonies per apiary x 4 sampling periods) samples were collected. Each sample was analyzed for 12 IA. In total, 4320 (360 x 12) elementary data were obtained.

The overall prevalence and the prevalence of each IA were assessed at apiary level then at colony level, as well as the most frequent co-infections occurring in colonies. Comparisons of the prevalence of each IA between colonies dying during or at the end of the study and colonies surviving were performed, using the Chi-square and Student's *t* tests when conditions of the tests were met. Presentation of the results was done using Microsoft Excel and SPSS® (Statistical Package for the Social Sciences) software, with the help of specially designed programs when necessary, developed using Delphi software.

RESULTS

The field observations performed in parallel to laboratory analyses allowed us to find that a majority of the colonies were "disease-free" and correctly withstood the beekeeping season (38 colonies out of 90 died after wintering).

PREVALENCE OF THE INFECTIOUS AGENTS IN THE APIARIES.

An apiary was considered infected if only one of its colonies was positive (infected) for one IA, whatever its load. All the apiaries of the study were infected by several IA, the number of IA found ranging from 6 to 12, the median being 9.

IA	Prevalence in colonies (%)	Prevalence in apiaries (%)	Percentage of infected colonies per apiary (%)		
			Average	Minimum	Maximum
<i>P. larvae</i>	66	99	65	35	90
<i>M. plutonius</i>	26	76	34	25	44
<i>N. apis</i>	5	29	10	10	10
<i>N. ceranae</i>	71	99	71	20	100
ABPV	4	14	24	20	28
BQCV	52	83	66	43	85
CBPV	54	90	67	45	100
DWV	84	96	88	65	100
IAPV	24	65	32	25	39
KBV	42	75	54	30	90
SBV	56	85	67	25	85
VdV1	56	94	61	39	90

TABLE I: Prevalence of infected colonies and infected apiaries during 2009 and average, minimum and maximum percentage of colonies infected by an Infectious Agent (IA) in the apiary. An apiary was considered infected if one of its colonies was positive (infected)

For each IA, the prevalence in the apiaries (number of apiaries infected among all apiaries) was calculated (Tab. I). During 2009, *P. larvae* was found in 99% of the apiaries, *M. plutonius* in 76%, *N. apis* in 29%, *N. ceranae* in 99%, ABPV in 14%, BQCV in 83%, CBPV in 90%, DWV in 96%, IAPV in 65%, KBV in 75%, SBV in 85% and VdV1 in 94% of the apiaries.

The proportion of infected colonies per apiary was very variable ranging from 10 to 100% (Tab. I).

PREVALENCE OF THE INFECTIOUS AGENTS IN THE COLONIES.

At the colony level, on average, more than five IA were detected per colony.

For each IA, the following prevalences were obtained (Tab. I): *P. larvae*, 66% of the colonies; *M. plutonius*, 26%; *N. apis*, 5%; *N. ceranae*, 71%; ABPV, 4%; BQCV, 52%; CBPV, 54%; DWV, 84%; IAPV, 24%; KBV, 42%; SBV, 56% and VdV1, 56% of the colonies.

During 2009 the most frequent co-infections detected in the colonies (found in more than 50% of the colonies) were DWV with either *N. ceranae*, or *P. larvae*, or SBV, or VdV1 or CBPV. The co-infections between bacteria, microsporidia and viruses were common in the colonies.

Besides these results of prevalence, most of the colonies among the 90 colonies studied were asymptomatic and when clinical signs were observed, they were punctual in space (one or two colonies among the five visited in each apiary) and in time (the signs were often no longer observed at the following visit).

INFECTION BY IA AND SURVIVAL OF COLONIES.

No specific pattern could be highlighted when comparing the prevalence of the IA between dying and surviving colonies (Fig. 2) (Chi-square = 7.2, df = 9, $P < 0.05$). Also, no significant difference was found in infectious loads of IA between dying and surviving colonies (Student's test: $t = 0.48$). No significant relation could thus be established between a given agent and the death of colonies during or at the end of the study.

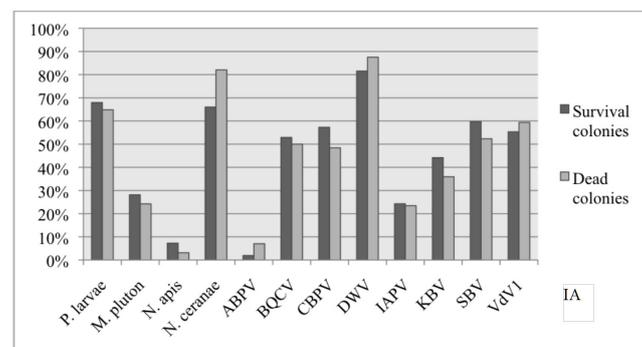


FIGURE 2: Comparison of the percentage of infected colonies by each Infectious Agent (IA), all sampling periods, depending on their fate during and after the study (survival / dead colonies)

DISCUSSION

PREVALENCE OF THE INFECTIOUS AGENTS IN THE COLONIES AND THE APIARIES.

At the apiary level, the prevalence of each IA was found to be high, except for *N. apis* and ABPV. All the apiaries were infected by at least six IA, including the *V. destructor*-free apiary (Ouessant IO).

The case of two agents needs to be further mentioned. First, it has to be noted that the apiary of Ouessant has always been considered as "DWV-free", even still in 2004 {34}, {35}. The absence of DWV on the island was then explained by the strict isolation of the island for several decades and by the absence of *V. destructor*, which is considered as the main vector of the virus {5}, {34}. However, in our study, the virus was found on this island, although no mites were detected, this assertion being based on the visits to apiaries of the island and controls carried out annually. Consequently, *V. destructor* is not the only factor of DWV transmission, in accordance with the study of Chen et al. {8} who had already detected the virus in hives uninfected with the mites. The classical PCR used for DWV study of Tentcheva et al. {34} could explain the lack of detection. Indeed, in our study, quantitative PCR was used, a very sensitive method allowing detection of very low viral loads. Similarly, VdV1, which had never been looked for on the island of Ouessant before, was found to be present on the island in our study. Our data give arguments for calling into question the involvement of *V. destructor* as a major factor of transmission of the virus {29}.

Depending on each IA, the number of colonies infected in each apiary was highly variable, from only a few colonies to all colonies infected. The horizontal transmission of the agents between colonies in the same apiary could be linked to several factors.

At the colony level, we can notice that between the two agents of foulbrood, *P. larvae* was more common than *M. plutonius*. Also, between the two agents of nosemosis, *N. ceranae* was much more present than *N. apis*. Concerning the viruses, ABPV was virtually absent and general viral infections seemed to be common in the colonies, DWV being the most frequent and one of the most abundant.

To compare these prevalence results, we referred to similar studies, i.e. carried out on randomly selected field colonies, and not a pool chosen for its particular problems (depopulation, mortality, etc.). Compared to this literature, the results appeared quite similar for the agents of American and European foulbroods {14}, {24} as well as for the agents of nosemosis {6}. Some differences were observed for the viruses, compared to the studies of Tentcheva et al. {34} and Ongus {29}, but they remained rather insignificant, except for CBPV, detected in 54% of the colonies in our study, while reported in only 9% of colonies in the study of Tentcheva et al. {34}. The results for the IAPV virus could not be compared with those of other studies, due to a lack of studies on the normal sanitary status.

Many co-infections were present in our study. *Nosema ceranae*, *Paenibacillus larvae* and DWV were found in most of the main co-infections. To our knowledge, these three agents, belonging to three different classes of infection (microsporidia, bacteria and viruses) had never been checked and thus never found together before, despite their frequent individual detection.

According to their high prevalence in the colonies, it would be interesting to know to what extent these three agents can potentially interact in the honey bee. However, if these agents are part of the normal health status of the colonies, it can be assumed that their interaction is very low. Therefore, in a wider scope, the potential interactions of these agents with other stressing factors of the bee colony, such as pesticides, would be interesting to develop. This has already been the case for some of them (e.g. between *Nosema* spp. and systemic insecticides: {1}, {38}).

The co-infections most frequently reported here were consistent with the co-infections found in diseased colonies, such as co-infections between ABPV and KBV {12}, {20}, ABPV and BQCV {3} ABPV and BQCV + / - SBV {36}, ABPV, DWV, and SBV {27}, KBV and SBV {33}, BQCV, DWV, SBV + / - KBV {7}, {27} BQCV and SBV {3}, DWV and VdV1 {29}.

According to our results, apiaries and colonies appear to be important reservoirs of infection, often infected by many different categories of agents (bacteria, microsporidia, viruses), but rarely affected by severe troubles such as depopulation, well-known clinical signs of major diseases, etc.,. We therefore gave them the status of asymptomatic carriers.

INFECTIOUS LOADS AND PATHOGENICITY.

After statistical tests, no link was found for any IA between their presence and the mortality of the colonies. Furthermore, no correlation was found neither between the colonies showing clinical signs and their loads in causative agent of the disease expressed (for a given disease, loads could be higher in asymptomatic colonies compared to diseased colonies), nor between the mortality of the colonies and their loads in IA.

These results made the complexity of the pathogenicity of the infectious agents explicit and showed the difficulty to establish pathogenicity thresholds of the IA of the honey bee. The presence of an agent in a colony does not necessarily mean that the larvae and/or adult bees will show clinical signs, or that the colony will die. The reason(s) for the death of the colony has/have to be looked for at another level.

A colony is a superorganism, an ecosphere continuously in balance with its viable colony count (species, strains, etc.) and its nutritional resources (quantity, diversity, quality). It is able to self-regulate its own system in case of imbalance and of responding to the fluctuations occurring in normal conditions. However many unfavorable factors, through repeated attacks, can weaken this capacity of self-regulation and consequently allow the proliferation of IA in asymptomatic carrier colonies and the outbreak of a disease {13}. These factors can be separated into two main categories: the intrinsic (virulence of the agent, contamination level of the colony, tolerance to the disease developed by the

bee population, co-existence with various other infectious agents and a high parasitic pressure by *Varroa destructor*) and extrinsic (beekeeping practices, use of the landscape, climatic conditions) factors.

All these factors are difficult to assess and/or control to ensure an accurate and complete analysis of the whole ecosystem with which the honeybee is interacting.

All these findings lead to confirm the hypothesis of a common asymptomatic carriage of infectious agents since an average of more than five IA can be found in a colony (among the 12 agents studied here). These infectious agents are commonly present in the bee without necessarily showing clinical signs, until an additional factor is added, causing the onset of clinical signs by exceeding the bees' tolerance level.

In conclusion, this study allowed us to obtain a representative idea of the current situation of the infectious status in apiaries and colonies for the western part of France. It reinforced the idea that good health of the colonies does not mean an absence of infectious agents, but continuously requires the control of the risk factors that may increase transmission and pathogenicity of these agents' supposed pathogens.

These results are useful both for recent research: they constitute a "control" point of comparison for studies performed on colonies suffering of depopulation; and also for future research: they give a temporal point of comparison for subsequent analyses in similar conditions.

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