Epidemiological study of honeybee pathogens in Europe: The results of Castilla-La Mancha (Spain)

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Abstract

As a part of a Pilot Monitoring Program of honey bee health coordinated by the EURL (European Union Reference Laboratory) and according to the criteria established for Spain, 14 apiaries in Castilla-La Mancha were selected at random and sampled during the autumns of 2012-2014 to identify the most prevalent nosogenic agents, potentially those related to the honey bee colony collapse phenomenon. In all the apiaries studied, Nosema ceranae was the most prevalent pathogen detected over the three years, confirming the worldwide spread of this microsporidan, a pathogen that negatively affects honey bee health at an individual and colony level. Trypanosomatids were also very prevalent in honey bee colonies, although the majority of Trypanosomatids detected were not Crithidia mellificae but rather the genetically distinct Lotmaria passim lineage. We also detected Varroa destructor mites, and the particularly high prevalence in 2014 suggests a possible problem regarding mite control in field conditions that requires attention. In agreement with data from other regions, the BQCV and DWV were the most prevalent viruses in honey bee colonies and thus, the Varroa-DVW interaction may be an important cause of bee colony mortality. While there was little evidence of a relationship between the BQCV virus and N. ceranae under field conditions during 2012, this was not the case in 2013 and 2014. Finally, the AKI-complex or LSV-complex was not detected. The information obtained in this study should help orientate future plans for honey bee disease control.

Additional key words: Apis mellifera; monitoring program; Nosema ceranae; Varroa destructor; Lotmaria passim; honey bee viruses.

Abbreviations used: AKI (Acute bee paralysis, Kashmir bee and Israeli acute paralysis complex viruses); BQCV (Black Queen Cell Virus); CLM (Autonomous Community Castilla-La Mancha); DWV (Deformed Wing Virus); EURL (European Union Reference Laboratory); LSV (Lake Sinai Virus); PCR (Polymerase Chain Reaction).

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Introduction

The honey bee, Apis mellifera Linnaeus, 1758, plays an essential role in the development cycle of a large number of plant species (Breeze et al., 2011) and it has produced food (honey and pollen) for human consumption for thousands of years (Chauzat et al., 2013). In the last decade, honey bees have been affected by serious health threats that have contributed to a substantial loss of honey bee colonies around the world (VanEngelsdorp & Meixner, 2010; Van der Zee et al., 2014). Persistent honey bee colony losses may be most problematic in areas like Spain, where professional beekeepers are essentially dedicated to honey production. Indeed, Spain is the country with the greatest honey production in Europe (more than 29 thousand tons: MAGRAMA, 2012, 2013), the largest number of honey bee colonies (more than 2.5 million on 01/05/2014: MAGRAMA, 2013) and the highest percentage of professional beekeepers (Chauzat et al., 2013). For this reason, honey bee colony losses are a major concern in this country. Castilla-La Mancha is...
one of the autonomous Spanish regions with the highest number of honey bee colonies (>155,000 colonies), and it is the fifth largest producer of honey in Spain. Indeed, “La Alcarria Honey” is produced in this area, the first honey to be awarded a European Protected Designation of Origin, highlighting the beekeeping tradition in the region and its essential impact on the agrarian structure in Spain.

A multitude of factors (viruses, mites, microsporidia, pesticides, etc.), either acting alone or in combination, have been associated with the worldwide decline in pollinators that causes premature colony mortality (Potts et al., 2010; Ratnieks & Carreck, 2010; Runckel et al., 2011; Cornman et al., 2012; Francis et al., 2013; Chen et al., 2014; Simon-Delso et al., 2014; Staveley et al., 2014; Porrini et al., 2016). However, the main drivers of colony collapse might differ in distinct geographical locations (Cepero et al., 2014, 2016). While the most prevalent nosogenic agents related to the phenomenon of honey bee colony collapse have already been studied in Spain (see for example: Martín-Hernández et al., 2007, 2012; Higes et al., 2009, 2010a; Bernal et al., 2010; Antúnez et al., 2012; Garrido-Bailón, 2012; Botías et al., 2013), it is vital to maintain a surveillance system to detect changes in the prevalence of such agents. In the present work, we report the prevalence of the major honey bee pathogens in 14 apiaries situated in the region of Castilla-La Mancha, and we investigated their potential role in the decline of honey bee health experienced in this area with such an important beekeeping tradition.

**Material and methods**

**Sampling protocol**

As part of the Pilot Monitoring Program of honey bee health coordinated by the EURL (European Union Reference Laboratory), 14 apiaries located in Castilla-La Mancha were selected at random and sampled during the autumn of 2012, 2013 and 2014 (September to November). The number of apiaries to be studied in this region was determined by the MAGRAMA criteria. In each apiary, a minimum of 8 and a maximum of 13 bee colonies were selected at random, and a total of 164 (2012), 151 (2013) and 8 (2014) worker honey bee samples were sent to the Centro de Investigación Apícola y Agroambiental (CIAPA) Honey Bee Pathology Laboratory (see Table 1 & Fig. 1). Each sample consisted of more than 300 adult worker bees for analysis and they arrived at our laboratory in perfect conditions (alive or frozen). Samples were taken during the mandatory application period of acaricides to varroosis control in Spain.

**Varroa destructor analysis**

To assess the presence of *Varroa destructor* Anderson & Trueman, 2000, in all worker honey bee samples from each colony in each apiary, we followed the EURL recommendations for the Epilobee program, adapted from the OIE (2008) methods. Each mite detected was collected individually and analyzed macroscopically to confirm the species, differentiating it from *Braula coeca* Nitzsch, 1818 or *Tropilaelaps clareae* Del-Finado & Baker, 1962. A honey bee colony was considered infested with *V. destructor* when at least one *Varroa* mite was found in the sample. The rate of infestation of the bee colony was estimated by assessing the number of *Varroa* mites in relation with the number of adult bees in each sample (more than 300 in all cases) and it was expressed as the number of *Varroa* mites/100 bees/sample.

**Nucleic acid extraction for pathogen detection**

Each of the honey bee colonies that were sampled during the study were analysed to detect the main pathogens. To obtain nucleic acids, a sub-sample of 120 worker honey bees from each honey bee colonies was used. The remaining bees (more than 180) were kept frozen at -80°C. Each sub-sample was macerated in AL buffer 50% (Qiagen) as described previously (Antúnez et al., 2012; Cepero et al., 2014, using sterile bags with a filter (BA6040 STRAINER BAGS) in a Stomacher 80 blender (Biomaster). The macerated bees were centrifuged at 3,000 rpm for 10 min, and the resulting pellets were used for DNA extraction and the supernatants for RNA extraction. Both the pellets and supernatants were stored at -20°C prior to nucleic acid (DNA or RNA) extraction.

For DNA extraction, the pellets were resuspended in 3 mL milliQ H₂O and a 400 µL aliquot was transferred to a 96-well plate (Qiagen) with glass beads (2 mm diameter, Sigma) using disposable Pasteur pipettes. After overnight pre-incubation with Proteinase K (20 µL, Qiagen), the samples were then processed as described previously (Martin-Hernández et al., 2012) and followed the BS96 DNA Tissue extraction protocol in a BioSprint station (Qiagen). The plates were then stored at -20°C.

For RNA extraction, 400 µL of the supernatant was incubated for 15 min with protease (20 µL, Qiagen) at 70°C and the nucleic acids were then extracted as described above (Biosprint 96 DNA; Qiagen and BioSprint workstation). The nucleic acids recovered were then subjected to DNA digestion with DNase I (Qiagen) to completely remove any genomic DNA and the total.
RNA recovered was used immediately to generate first strand cDNAs using the Quantitec Reverse Transcription Kit (Qiagen) according to the manufacturer’s instructions. The resultant cDNA was used for subsequent virus analysis (with no further dilution).

Negative and positive controls were run in parallel for each step: bee maceration, DNA and RNA extraction, and reverse transcription.

**PCR and RT-PCR reactions**

Broad pathogen screening (Table 2) was performed using published PCR assays to detect: *Acarapis woodi* (Rennie, 1921; Cepero et al., 2015); *Nosema apis* (Zan-der, 1909) and *Nosema ceranae* (Fries et al., 1996) in triplex PCR with an internal control (Martín-Hernández et al., 2012); Trypanosomatids and Neogregarines (Meeus et al., 2010). Virus analysis was performed using RT-PCR published to detect Lake Sinai Virus complex (LSV1-LSV2-complex; Ravoet et al., 2013); Acute Bee Paralysis Virus-Kashmir Bee Virus-Israeli Acute Paralysis (AKI-complex: Francis & Kryger, 2012); Black Queen Cell Virus (BQCV: Bailey & Woods, 1974) and Deformed Wing Virus (D WV: Bailey et al., 1979) using qRT-PCR described by Chantawannakul et al., 2006). Negative PCR controls were included in all analyses.

**Trypanosomatid sequencing**

To identify the infecting species, PCR products of all trypanosomatid positive isolated were sequenced. For this, the PCR products were purified (Gómez-Mora-cho et al., 2015) and sequenced on an ABI3730XL Automatic Sequencer using Big Dye (Applied Biosystems, Foster City, CA, USA). The resulting sequences were assembled, aligned and checked for accurate base calling using CodonCode Aligner (CodonCode Corporation, Dedham, MA, USA). The species were identified based on a single nucleotide variant that allows the 18S rDNA sequences of *L. passim* (Schwarz et al., 2015) and *Crithidia mellificae* (Langridge & McGhee, 1967) to be discriminated (“G” for *L. passim* and “A” for *C. mellificae*, respectively: Cepero et al., 2014, 2016), and also compared with sequences deposited in GenBank database.

**Statistical analysis**

Statistical analyses were performed using the SPSS 23 software, carrying out Fisher Exact Tests to determine if there was any association between the pathogens. Chi-square significance tests were also performed to check if the prevalence of each pathogen changed over the years and if there were any pathogen associations.
Results

The prevalence of the major bee pathogens is shown in Table 3. The most prevalent pathogen over the three years of sampling was *N. ceranae*, with a mean prevalence of 52.6% in all the apiaries sampled (53.7%, 40% and 64.2% in each sampling period) and present in all apiaries studied). Trypanosomatids were also detected in all the apiaries analysed, with a mean prevalence of 32.6% (29.9%, 27.1% and 40.7%, in each year). The sequencing of all positive trypanosomatid isolates indicated that *L. passim* was the most abundant in this area since 80% of the analysed sequences corresponded with this species, whereas *C. mellificae* was far less frequent (20% of sequences studied).

*V. destructor* was the third parasite in terms of prevalence, with a mean prevalence of 18%. *V. destructor* was identified in three of the five provinces in 2012 (Albacete, Guadalajara and Toledo) but in only 4 of the 14 apiaries analysed (28.6%), with a mean prevalence of 9.1% and a mean percentage parasitization of 0.4% in this year. However, its prevalence increased significantly in the next two years, with an average prevalence of 11.9% in 2013 and 33% in 2014. *V. destructor* was detected in all the apiaries studied in 2013 and 2014. The mean percentage of parasitization increased in 2013 to 1%, and rose to 3% on average in 2014. *N. apis* was the fourth parasite in prevalence, with a mean of 5.6%. It was not detected in 2012, but in 2013 and 2014 its prevalence was 15.2% and 1.6% respectively. Neogregarines were the fifth parasite in prevalence, with a mean prevalence of 1.6% (0.6%, 4.6% and 0% in each year).

Of the seven viruses analyzed, only DWV and BQCV were found in all the apiaries analysed. The mean prevalence for BQCV was 25.1% (30.5% in 2012, 23.8% in 2013 and 21.1% in 2014). For DWV the mean prevalence was 18.6% (14.6% in 2012, 11.9% in 2013 and 29.3% in 2014). No LSV or AKI viruses were found.

Regarding associations, a higher presence of DWV was observed in the colonies where *V. destructor* was detected (Chi-squared; *p*<0.0001), whereas BQCV was less common in these colonies (Chi-squared; *p*<0.05). The detection of BQCV was associated with the presence of *L. passim* in honey bee colonies (Chi-squared; *p*<0.05 in 2012, and with *N. ceranae* in 2013 and 2014 (Chi-squared; *p*<0.05). The detection of *N. ceranae* in honey bee colonies was not significantly associated with the appearance of *L. passim* (Chi-squared; *p*>0.05).

### Table 2. Primers used for each pathogen in PCR reactions.

| Pathogen     | Primer   | Sequence                        | Amplicon size (bp) |
|--------------|----------|---------------------------------|--------------------|
| *N. ceranae* | CER-F    | 5’-CGGCGACGATGATGAAATATTAA-3’   | 218-219            |
|              | CER-R    | 5’-GGGGGATGCTTTTACAGTATAATTTAA-3’ | 321               |
| *N. apis*    | CER-F    | 5’-GGGGGATGCTTTTACAGTAAATTTAA-3’ | 417               |
| *Trypanosomatids* | CRI-SEF  | 5’-CCTTTGTCGGTGAGGTGAT-3’      | 260               |
| *Neogregarines* | API-NEOF  | 5’-CCACCAAGGAAGACATGAAAG-3’    | 180               |
| *A. woodi*   | AW180-FOR| 5’-GAAAATGACGTGTTTAGGTGTC-3’   | 250               |
| *RT-PCR*     | LSVdeg-F | 5’-GCCWCRGTYTTGGTYCCCMCC-3’    | 600               |
| *AKI*        | AKI-F    | 5’-CCTTCTGATGAGAATCTC-3’       | 100               |
| *RT-qPCR*    | BQCV 9195F| 5’-GGTGGCAGGAGATGATGGA-3’      | 305               |
| *DWV*        | DWV 9587F| 5’-CCTGAGAAGCTTCTCGTGAAGA-3’   | 250               |
|              | DWV 9711R| 5’-ATTCAGGACCCACCAATCCATG-3’   |                    |

*5’ (FAM, 6-carboxyfluorescein); 3’ (TAMRA, tetramethylrhodamine)*
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Table 3. Prevalence of the main bee pathogens per year.

| Pathogen         | 2012 |          |          | 2013 |          |          | 2014 |          |
|------------------|------|----------|----------|------|----------|----------|------|----------|
|                  | Positive samples | % | Positive samples | % | Positive samples | % |
| V. destructor     | 15   | 9.1      |          | 18   | 11.9     |          | 41   | 33.3     |
| N. ceranae       | 88   | 53.7     |          | 60   | 39.7     |          | 79   | 64.2     |
| N. apis          | 0    | 0        |          | 23   | 15.2     |          | 2    | 1.6      |
| Neogregarines    | 1    | 0.6      |          | 7    | 4.6      |          | 0    | 0        |
| Tripanosomatids  | 47   | 29.9     |          | 41   | 27.2     |          | 50   | 40.7     |
| A. woodi         | 0    | 0        |          | 0    | 0        |          | 0    | 0        |
| BQCV             | 50   | 30.5     |          | 36   | 23.8     |          | 26   | 21.1     |
| DWV              | 24   | 14.6     |          | 18   | 11.9     |          | 36   | 29.3     |
| LSV1 and 2       | 0    | 0        |          | 0    | 0        |          | 0    | 0        |
| AKI-complex      | 0    | 0        |          | 0    | 0        |          | 0    | 0        |

Discussion

As the EURL reports, the Epilobee project requires a strong effort to coordinate and collaborate with beekeepers in order to correctly sample and analyse honey bees. This study formed part of this project, delivering scientifically solid data on honey bee health in a particular geographical area within the EU. Indeed, we report here the prevalence of the major honey bee pathogens in Castilla-La Mancha that are potentially related to the weakness and mortality of honey bee colonies evident in Europe since the early years of the 21st century.

As reported previously for Spain (Higes et al., 2009, 2010a; Botías et al., 2012; Martín-Hernández et al., 2012; Cepero et al., 2014, 2016), N. ceranae was the most prevalent pathogen over the three years in all the apiaries studied. This high prevalence was also found in other parts of Europe (Stevanovic et al., 2011; Ravoe et al., 2013), in South (Martínez et al., 2012) and North America (Runckel et al., 2011; Martín et al., 2013; Emsen et al., 2016), and in Asia (Li et al., 2012; Morimoto et al., 2013; Yang et al., 2013), confirming the worldwide spread of this microsporidian (Martín-Hernández et al., 2007; Higes et al., 2010a,b, 2013). The role of N. ceranae in honey bee colony loss remains controversial (Higes et al., 2013). Yet despite this dispute, it is well accepted that N. ceranae negatively affects honey bee health at the individual and colony levels (Higes et al., 2008, 2009; Martín-Hernández et al., 2009; Dussauba et al., 2012, 2013; Ravoe et al., 2013; Gobliirsch et al., 2013; Botías et al., 2013; Alaux et al., 2014; Cepero et al., 2014, 2016; Vidau et al., 2014; Wolf et al., 2014; Maes et al., 2016; Bordier et al., 2017), causing colony collapse under certain conditions (Higes et al., 2013; Betti et al., 2014). Thus, this pathogen clearly represents a serious risk for professional beekeeping in temperate areas of the world (Higes et al., 2009; Hatjina et al., 2011; Nabian et al., 2011; Martín-Hernández et al., 2012; Lodesani et al., 2014; Adjlane & Haddad, 2016). An additional problem with N. ceranae infection is that this pathogen can spill over from honey bees into bumble bee populations, causing fatal infection and contributing to bumble bee decline (Plischuk et al., 2009; Graystock et al., 2013; Fürst et al., 2014). Therefore, N. ceranae poses a threat to managed and wild pollinators, and developing control strategies against N. ceranae is now a priority in contemporary bee research in order to save both honey bees and wild bees.

Trypanosomatids were also very prevalent in honey bee colonies, mainly L. passim. These pathogens are currently receiving much attention given that the presence of C. mellificae has been correlated with colony losses in the USA and Belgium, especially when detected in conjunction with N. ceranae (Runckel et al., 2011; Cornman et al., 2012; Ravoe et al., 2013). Such coinfection was also reported in depopulated honey bee colonies (Cepero et al., 2014), although the presence of N. ceranae was not significantly associated with the detection of L. passim in the honey bee colonies studied here, suggesting that this relationship may only occur in conjunction with colony collapse. When these Trypanosomatids were sequenced for species identification, we confirmed the previous data (Cepero et al., 2014) indicating that the majority of Trypanosomatids detected in honey bees were not C. mellificae but rather, the genetically distinct lineage, L. passim (Schwarz et al., 2015).
**V. destructor** mite is a serious and devastating ectoparasite of honey bees worldwide (OIE, 2008; Rosenkranz et al., 2010) that is strongly suspected to participate in the collapse of bee colonies (Dainat et al., 2012; Francis et al., 2013). In the present study we detected *Varroa* mites in three of the five provinces in the first year (2012), yet its prevalence increased, especially in 2014. In Spain there is legislation on the management of varroosis (BOE, 2006) that requires the application of a veterinary product to control this mite during autumn. Based on the results obtained here, it appears that this control was effective in the area studied during 2012 and 2013, although the increase in prevalence detected in 2014 indicated a problem in mite control in field conditions, situation that has worsened from 2015 to the present, as our team has been able to confirm based on the data that our passive surveillance system shows in this regard.

Gregarines are a diverse group of apicomplexa protoists that infect many invertebrate phyla (Stejskal, 1965). The neogregarine *A. bombi* (Liu, Macfarlane & Pengelly, 1974) is considered to be an infrequent parasite of *Bombus* species and although the use of a molecular detection method has led to its detection in honey bees (Meeus et al., 2010; Plischuk et al., 2011; Maharroamov et al., 2013; Morimoto et al., 2013; Cepero et al., 2014), the potential pathological effect of *A. bombi* in honey bees is not clear. Indeed, it has even been suggested that the PCR primers used to detect this protist (Meeus et al., 2010) may also amplify other pathogens, which should be taken into account to avoid misdiagnosis (Cepero et al., 2014). The results obtained here indicate that as reported previously (e.g., Plischuk et al., 2011; Marimoto et al, 2013), Neogregarines have a very low prevalence in Castilla-La Mancha and thus, they would not be a great threat for local bee colonies if pathogenic.

Honey bees can be infected by as many as twenty RNA viruses, of which twelve have been detected in Europe (Aubert et al., 2008). The most frequent are the AKI-complex (Francis & Kryger, 2012; Francis et al., 2013), BQCV and DWV (Runckel et al., 2011; Mondet et al., 2014), and LSV-complex mainly LSV1 and LSV2 (Runckel et al., 2011; Ravoet et al., 2013) which could therefore be related to bee colony collapse. In agreement with data from other regions (Chen & Siede, 2007; Teixeira et al., 2008; Li et al., 2012; Antúnez et al., 2012; Morimoto et al., 2013; Ravoet et al., 2013; Rodriguez et al., 2014), our results show that BQCV and DWV were the most prevalent viruses in Castilla-La Mancha. The *Varroa*-DWV interaction is thought to be an important cause of mortality in bee colonies (Rosenkranz et al., 2010; Dainat et al., 2012; Francis et al., 2013) and our study confirmed this relationship as the presence of *V. destructor* was positively correlated with the occurrence of DWV.

In the field, BQCV disease outbreaks have been linked with infection with *N. apis*. The rapid multiplication of BQCV in adult bees infected with this microsporidian (Bailey, 1981, 1982) might be a secondary effect of such infection, either by increasing the susceptibility of the alimentary tract to infection by this virus (Chen & Siede, 2007) or by activating its replication in the gut where *N. apis* resides (Chen et al., 2006). Given that the gut is also the target organ for infection by *N. ceranae*, the same relationship might be expected between this microsporidian and the BQCV virus. Such an association was indeed reported recently (Dainat et al., 2012; Mendoza et al., 2014; Francis et al., 2014) and although our data did not support this relationship under field conditions in 2012, they did in 2013 and 2014. While a positive association between *Nosema* infection and BQCV has been found in some studies, the mechanism by which *Nosema* activates and transmits BQCV infection remains to be determined (Chen & Siede, 2007).

The AKI-complex and LSV-complex were not detected here and while IAPV has been related to honey bee colony collapse (Cox-Foster et al., 2007; Chen et al., 2014), its prevalence in Spain was very low (Garrido-Bailón et al., 2010; Antúnez et al., 2012) as confirmed here. The abundance of a new virus complex in honey bees (Lake Sinai viruses complex, LSV-complex: Runckel et al., 2011; Granberg et al., 2013; Ravoet et al., 2013; Cepero et al., 2014) suggests that this group may play significant role in colony health (Runckel et al., 2011, Cormman et al., 2012; Cepero et al., 2014). However, this hypothesis is not always supported (Ravoet et al., 2013) and we did not detect this complex here.

In conclusion, in accordance with previous studies in Spain (Higes et al., 2009; Garrido-Bailón et al., 2012; Martín-Hernández et al., 2012), the most prevalent pathogens in Castilla-la Mancha are *N. ceranae, L. passim*, BQCV and DWV. Future disease control should focus on these pathogens and the effects that the interactions between them could have on bee colony health. Another priority should be to monitor changes in prevalence to detect potential problems with pathogen control programs, such as those that possibly occurred in 2014 with the *Varroa* control program that was apparently effective in field conditions until then. As such, studies like this will help advance our understanding of pathogen interactions and bee health, therefore helping in the application of control measures to limit colony losses.
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