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# Analytical sensitivity and specificity of a RT-PCR for the diagnosis and characterization of the spatial distribution of three *Apis mellifera* viral diseases in Spain\*

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**Abstract** – The occurrence and spatial distribution of deformed wing virus (DWV), black queen cell virus (BQCV), and Kashmir bee virus (KBV) were assessed in 294 honeybee colonies in Spain by employing a SYBR-Green based real time RT-PCR. 60% of them were positive for both DWV and BQCV, and those two viruses were detected in 84% and 68% of the samples, respectively. Conversely, KBV was detected in only 1.7% of the samples. Increments in the number of bee colonies per region, adjusted by the number of samples collected, were associated with increased risk of finding DWV, BQCV, and KBV, as estimated by mixed Bayesian regression models. The residual risk for DWV, BQCV, and KBV decreased northerly and westerly, suggesting that factors or forces that favour the presence of these viruses could be more prevalent in southern and eastern regions of Spain. Results will be useful in the design and implementation of effective honeybee viral disease control and surveillance programs in Spain.

real time RT-PCR / *Apis mellifera* / RNA viruses / associated risk

## 1. INTRODUCTION

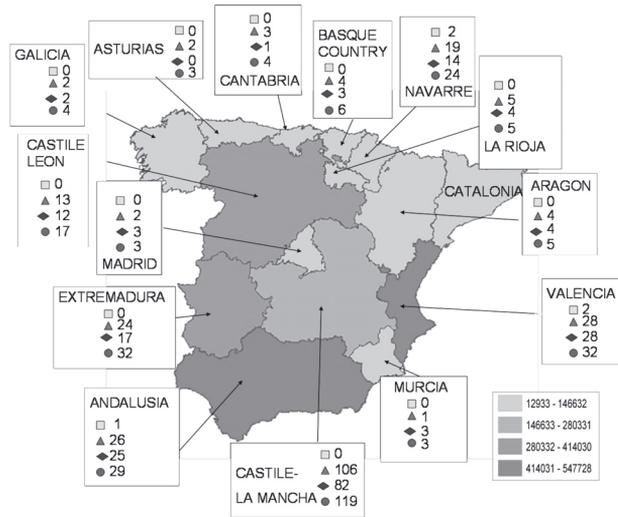
Spain is considered the third largest honeybee (*Apis mellifera* L.) producer country in the world (MAPA, 2004). Honeybee production has notably flowered since the early 1990s in Spain, including a 50% increase in the number of professional bee keepers in the last 15 years (MAPA, 2004). Much of the Spanish honeybee production takes place in the regions of Andalusia, Valencia, Castile-Leon, Extremadura, Castile-La Mancha, and Aragon, where 83.1% of the 2 464 601 bee colonies in the country are located (Fig. 1). The economic value of the

main honeybee-derived products (i.e., honey, pollen, and wax) has been estimated to represent well over EUR 60 million for Spain (MAPA, 2004).

Spanish honeybee production reached a peak in 2003, when 32.665 Tm were produced. Honeybee production, however, has substantially decreased from 2003 through 2005 as a consequence of persistent droughts, insufficient feeding, a crisis in the honeybee market, and emergence of new honeybee diseases (MAPA, 2004). It has been estimated that the combination of those factors has resulted in financial losses of EUR >50 million during the last 7 years (MAPA, 2004). This figure did not consider, however, indirect losses, like that one attributable to decrease in crops pollination.

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**Figure 1.** Number of bee colonies per region in Spain. Starting from the region with the lowest number of bee colonies (Cantabria = 12 933 bee colonies) to the region with the largest number of bee colonies (Andalusia = 547 728). The total number of samples collected per region (red circle), and samples positive to Kashmir bee virus (KBV, yellow square), to deformed wing virus (DWV, green triangle), and to black queen cell virus (BQCV, purple rhombus) detected on a survey conducted from 2004 through 2006 are indicated. No samples were obtained from Catalonia. Insular regions of Spain (Canary and Balearic Islands), where samples have not been obtained, are not shown.

Diseases caused by the deformed wing virus (DWV), black queen cell virus (BQCV), and the Kashmir bee virus (KBV) are considered among the most important viral diseases of honeybees in terms of the epidemiological and economic consequences of infected apiaries. BQCV infection is typically associated with mortality rates in pupae, and it is considered to be triggered by *Nosema apis* Zander infestation (Bailey and Woods, 1974; Bailey et al., 1983; Allen and Ball, 1996). Although the disease is more prevalent in queen pupae and worker pupae, adult bees are also susceptible to BQCV infection (Chen et al., 2004). Clinical manifestation of DWV infection ranges from asymptomatic or mild disease to an extensive repertoire of signs that include wing deformities, bloated abdomens, paralysis, and shorter adult lifespan of worker and drone bees. DWV infection has been associated with high levels of *Varroa destructor* Anderson & Trueman parasitism (Bowen-Walker et al., 1999; Nordström, 2003). Bees infected with KBV do not develop specific symptoms, and it has been suggested that KBV is the

most virulent known honeybee virus (Bailey et al., 1981; Allen and Ball, 1995). Incidentally, KBV infection has been associated with losses of hair from the thorax, darker colour of the bees, ataxia, and death (Allen and Ball, 1995). Although information on the prevalence of major honeybee viral diseases is uncertain and mostly anecdotal, estimates from surveys conducted in France, Uruguay, Austria, and Hungary suggest that they could be as high as 76–100% for DWV and 30–91% for BQCV (Tentcheva et al., 2004; Antunez et al., 2006; Berenyi et al., 2006; Forgách et al., 2007). Conversely, KBV appears to be much less prevalent than DWV and BQCV, with failure to identify the virus on surveys conducted in Uruguay, Austria, and Hungary (Antunez et al., 2006; Berenyi et al., 2006; Forgách et al., 2007). A notorious exception is France, where KBV was detected on 17% of the assessed samples (Tentcheva et al., 2004).

The presence of KBV, BQCV, and DWV has been reported in Spain (Allen and Ball, 1995; Higes et al., 2007; Kukielka et al., 2008), suggesting that infection by these

pathogens might be responsible at least in part for the decrease in honeybee production suffered by the country during the last five years. Prerequisites for controlling honeybee viral infections in Spain are the development of highly specific-highly sensitive diagnostic assays and knowledge on the extent of the distribution of the diseases throughout the country. Availability of a reliable diagnostic assay is a sine qua non condition for controlling a disease because it represents one of the major pillars of a sustainable disease-surveillance system. Knowledge on the spread of DWV, BQCV, and KBV in Spain is important to obtain preliminary evidence of the impacts that these diseases have on the honeybee industry of the country. It will also help to design and implement disease control and prevention programs by identifying the regions with the highest risk for a given disease and decreasing the risk for disease spread into regions where the disease is less prevalent. Moreover, quantitative knowledge on factors positively or negatively associated with disease prevalence could be useful to guide the allocation of human and financial resources as part of a disease control program. For example, one would hypothesize that regions with the largest number of bee colonies will also have the highest prevalence and risk for infectious honeybee disease introduction and spread as a consequence of the risk imposed by the presence of a large number of susceptible individuals in the region. Following this reasoning, resources for surveillance and control activities might be selectively allocated into regions with the largest number of bee colonies in order to control the disease in areas considered to be more susceptible to honeybee disease infection. To the best of our knowledge, the spatial distribution and the extent of association between DWV, BQCV, or KBV and number of bee colonies have never been quantified in Spain.

The goal of this paper was to estimate the analytical sensitivity and specificity of RT-PCR-based procedures aimed at identifying DWV, BQCV, and KBV infection in Spanish bee colonies. The paper presents preliminary evidence of DWV, BQCV, and KBV spatial distribution in Spain as well as estimates of the

spatial distribution of unmeasured factors and forces associated with disease distribution.

## 2. MATERIALS AND METHODS

### 2.1. Samples collection and processing

Samples from 294 bee colonies located in 14 of the 15 regions in which continental Spain is administratively divided were collected from 2004 through 2006 (Fig. 1). Each sample was obtained from a different beekeeper; some of the samples were located in the same geographical area, but no more than one sample from the same bee colony was analyzed. At the time of the sample collection, most (69.38%) of the bee colonies presented a wide range of clinical signs compatible with infection by at least one of the viral diseases assessed here. Recorded clinical signs included bloated abdomens, disorientation, and weakness, which ultimately led to the depopulation of the colony. Honeybees were taken from the hives and immediately ( $< 5$  r) frozen at  $-20$  °C until processing. Processing consisted of the sequential homogenization, centrifugation, and RNA extraction from the samples. Homogenization was achieved by crushing the sampled honeybees in a mortar with PBS solution in proportion of ten bees per 5 mL of PBS. Homogenized samples were centrifuged for 1.5 min at  $400 \times g$  and supernatant was collected and stored at  $-80$  °C. Homogenized and centrifuged samples were kept in sterile Eppendorf tubes and treated with TRIzol reagent following the manufacturer instructions for RNA extraction. Extracted RNA was resuspended in RNase free water and maintained at  $-20$  °C.

### 2.2. Real time RT-PCR for viruses

Presence of DWV, BQCV, and KBV in the processed samples was assessed by amplification of virus-specific nucleic acid using a RT-PCR. One step real time RT-PCRs based on SYBR-Green were developed for DWV and BQCV detection (Kukielka et al., 2008) and for KBV detection (Stoltz et al., 1995), which is a conventional RT-PCR that was adapted to real time by the authors. All reactions were performed in a Stratagene Mx-Pro 3000 using the Master mix and enzymes provided by Stratagene. The final amplified product consisted of a volume of 25 microlitres ( $\mu$ L) that included 2  $\mu$ L of RNA template, 12.5  $\mu$ L of SYBR-Green Master mix (Quantitative RT-PCR Brilliant

SYBR-Green Master Mix, Stratagene®), 0.0625 µL of StrataScript RT/Rnase Block Enzyme Mixture (Stratagene®), 0.3 µM of primers for BQCV detection, 0.2 µM of primers for DWV detection, and 0.4 µM of primers for KBV detection. The thermocycler program for DWV and BQCV was set up to run for 30 min at 48 °C (RT), followed by 10 min at 95 °C, 40 amplification cycles of 45 s at 95 °C, 1 min at 61 °C, and 1 min at 72 °C. The final extension was carried out during 7 min at 72 °C. The dissociation curve was estimated from 70 °C through 99 °C and running 40 cycles with increments of 1 °C every 30 s. The program for KBV was set up to run 30 min at 50 °C (RT) and 10 min at 95 °C, followed by 40 amplification cycles at 95 °C for 1 min, at 57 °C for 1 min, and at 72 °C for 1 min. The final extension and estimation of the dissociation curve was conducted using the same procedure described for BQCV and DWV. Primers used to perform the assays are shown in Table I.

The analytical specificity of the RT-PCR assay was evaluated by sequence analysis of size-specific amplification products. The real time RT-PCR products were purified using QIAquick PCR purification Kit (Qiagen) and analyzed by the sequencing services of SECUGEN S.A (Madrid, Spain). The sequence data of each virus fragment was analyzed using the BLAST® server at the NCBI. Amplified fragments of each virus were compared with reference sequences of the same virus published at the GenBank using BLAST® (blastn) and Clustal W® softwares. High percent sequence identity (> 94%) between GenBank sequences and sequences of the viruses isolated here was considered further evidence of high test specificity.

Due to the difficulty to obtain positive reference samples for honeybee viruses other than DWV, BQCV, and KBV, the absence of potential primer cross-reaction was estimated by running the complete genome sequences of other three honeybee viruses (Sacbrood virus, SBV; Chronic bee paralysis virus, CBPV and, Acute bee paralysis virus, ABPV) and the designed primers in the Clustal W® software. No matches between primers sequences and the complete genome of these other viruses were found. The specificity of the assays was given by the melting point analysis of the amplification products and by the determination of the nucleotide sequences. The analytical sensitivity of the method was investigated by testing ten-fold dilutions of selected positive sequenced samples for DWV, BQCV, and KBV and comparing them to the results of the conventional RT-PCR.

### 2.3. Data analysis

The extent of the association between number and density of apiaries and prevalence was quantified at a regional level using mixed Bayesian regression models that are modifications of those presented by Besag et al. (1991), Lawson and Zhou (2005), Stevenson et al. (2005), and Branscum et al. (2008), among others. The number of bee colonies infected by disease  $i$  was assumed to follow a Poisson distribution with mean  $\mu_i$ . The expected number of disease  $i$ -positive bee colonies ( $E_{i,j}$ ) on each of the 14 regions  $j$  where samples were obtained (Fig. 1) was computed as:

$$E_{i,j} = P_i \times n_j$$

where  $P_i$  is the prevalence of disease  $i$  in Spain, which was approximated here by estimating the proportion of where disease  $i$  was detected in the assessed bee colonies, and  $n_j$  is the number of bee colonies assessed in region  $j$ . Thus,  $E_{i,j}$  is an indicator of the number of bee colonies in each region  $j$  where disease  $i$  was expected to be found under the null hypothesis of spatially homogeneous distribution of cases. Let

$$\mu_{i,j} = E_{i,j} \times R_{i,j} \quad (1)$$

so that  $R_{i,j}$  quantifies the increase (or decrease) of risk for disease  $i$  in region  $j$ , compared with the background or average risk of the country, which is given by  $E_{i,j}$ . Therefore,  $R_{i,j} = 1$  indicates that the risk for disease  $i$  in region  $j$  is equal to the background risk of the country, and values of  $R_{i,j}$  higher or lower than 1 indicate that the risk in region  $j$  is, respectively, higher or lower than the background risk of the country. The log transformation of Equation (1) was:

$$\log(\mu_{i,j}) = \log(E_{i,j}) + R_{i,j}$$

and therefore,  $R_{i,j}$  was modelled using a linear regression function of the form:

$$R_{i,j} = \alpha + \beta \times B_j + U_{i,j} \quad (2)$$

where  $B_j$  is the number of bee colonies in region  $j$ ,  $\beta$  is the regression coefficient associated with the number of bee colonies in region  $j$ , and  $U_{i,j}$  is a random effect used to quantify the contribution attributable to factors or forces that have not been assessed in the model. The deviances from the background risk of the country for disease  $i$  associated with the number of bee colonies ( $RB_i$ ) and with

non-assessed unstructured factors ( $RU_i$ ) were estimated, respectively, as  $\exp(A_i)$  and  $\exp(U_i)$ . The deviance inference criterion (DIC) was computed to estimate whether inclusion of density of bee colonies per region as a risk factor into the model improved the model fitness, compared with the inclusion of  $B_i$ . Models with the lowest DIC value were assumed to provide the best model fitness (Spiegelhalter et al., 2002).

One of the diseases assessed here, KBV, was detected in only three regions. Therefore, for the case of KBV, regions were categorized as cases and controls, depending on whether the disease was detected or not. The probability of a region being a case was modelled using a mixed Bayesian logistic regression model of the form described in equation (1) and the values of  $RB_i$  and  $RU_i$  were computed using the same procedure described for the Poisson regression model.

Non-informative priors were used in the computations;  $U$  was assumed to follow a gamma distribution with parameters 0.5 and 0.0005, and  $\alpha$  and  $\beta$  were assumed to be normally distributed with mean = 0.01 and precision = 0.00001. All variables were centered by subtracting the mean value of the variable to the observed values and dividing the result by the standard deviation of the variable. Models were run using the WinBugs software (Spiegelhalter et al., 1996).

### 3. RESULTS

A total of 294 bee colonies distributed throughout Spain were assessed by SYBR-Green based real time RT-PCR for the presence of KBV, DWV, and BQCV, which are the most important honeybee viruses identified in Spain.

DWV was the most prevalent disease in the assessed samples (84.0%; CI95%: 79.8–88.2%), followed by BQCV (68.0%; CI95%: 62.7–73.4%), and KBV (1.7%; CI95%: 0.2–3.2%). DWV, BQCV, and KBV infection was detected in 14, 13, and 3 of the 14 regions where samples were collected, respectively (Fig. 1). The region of origin was missing in 8 out of the 294 samples. From these eight samples, eight, two, and none were, positive for DWV, BQCV, and KBV respectively. The confidence intervals for the proportion of pos-

itive results in the set of samples for which information on the origin was missing overlaps for the three diseases with the confidence intervals estimated for the samples where the origin was known, suggesting that missing data was unbiased. The eight samples for which the region of origin was unknown were eliminated from the regression models. The proportion of samples positive to at least one of the viruses found in bee colonies with clinical signs of disease (193/203) was similar (Fisher's test,  $P = 0.35$ ) to the proportion of positive samples in bee colonies without clinical signs of disease (89/91).

A large majority of the bee colonies were found to be infected by at least one of the viruses assessed here. Co-infection with two viruses, mostly between BQCV and DWV, was detected in 60.4% of the samples. Co-infection with the three viruses was detected in all the samples where KBV was detected.

The real time RT-PCRs were able to detect viral RNAs at dilutions as low as  $10^{-9}$  for DWV and BQCV, and as low as  $10^{-5}$  for KBV. Alignment of the sequences of the viruses isolated here with reference sequences for the same virus published at the GenBank showed an identity of 94–100%. No cross reaction was found when designed primers were aligned with the complete genome of the three viruses (SBV, CBPV, and APBV) selected for comparison.

These findings suggest that each assay was able to specifically discriminate infection with the virus for which it was designed from infection with other honeybee viruses.

Increments of one standard deviation (SD = 189 826) in the number of bee colonies per region were estimated to result in 3%, 12%, and 74% increments in the risk of finding, respectively, DWV (RB = 1.03, CI95% = 0.89–1.20), BQCV (RB = 1.12, CI95% = 0.95–1.32), and KBV (RB = 1.74, CI95% = 0.69–4.32). When compared with the number of bee colonies per region, inclusion of density of bee colonies per region as a risk factor into the model resulted in largest values of the DIC, suggesting a poorer fit of the models.

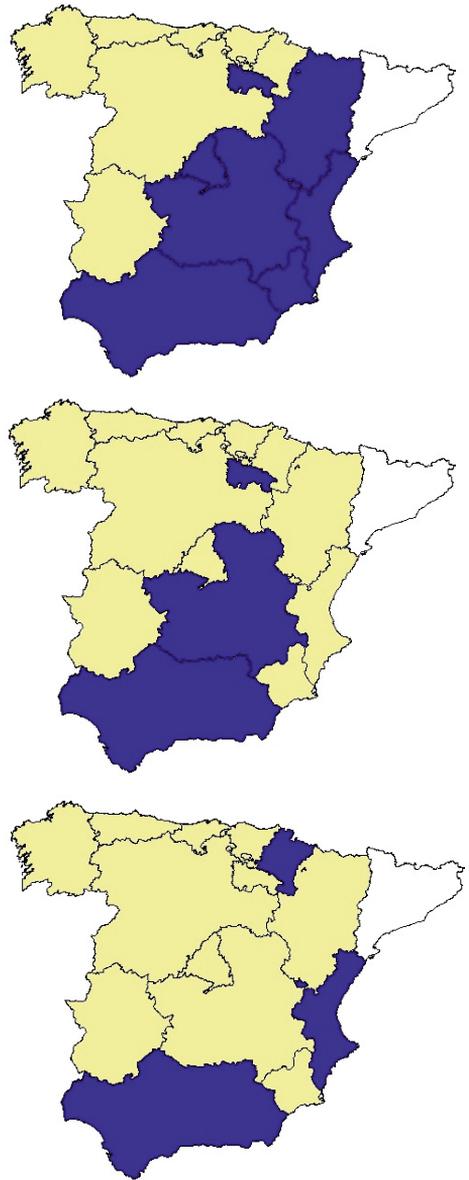
Visual inspection of the disease-specific residual risk remaining after accounting for the number of samples obtained and for the

number of bee colonies on each region suggests that the influence of non-assessed factors or forces that may lead to an increase in the risk for DWV, BQCV, and KBV tend to increase east- and southwards in Spain (Fig. 2).

#### 4. DISCUSSION

Spanish honeybee industry has been affected during the last seven years by a progressive disappearance of bees. Several factors are likely to be responsible for the observed high rate of bee mortality (Martín-Hernández et al., 2007) including infection by several viruses, some of which have recently been isolated in Spain (Kukielka et al., 2008).

Presence of DWV, BQCV, and KBV has been determined analyzing the samples using SYBR-Green based real time RT-PCRs. Several RT-PCR methods have been developed and applied for the diagnosis of eight bee viral infections (Stoltz et al., 1995; Benjeddou et al., 2001; Grabensteiner et al., 2001; Bakonyi et al., 2002; Ribière et al., 2002; Tentcheva et al., 2004; Chen et al., 2005; Genersch, 2005; Topley et al., 2005; Yue and Genersch, 2005; Chantawannakul et al., 2006; Blanchard et al., 2007; Maori et al., 2007). However, to our knowledge, this is the first time that a SYBR-Green based technique is described for the identification of honeybee viruses. The SYBR-Green based real time RT-PCR is a convenient method for the detection of honeybee viruses because of the simplicity and low cost of the procedure. The use of a highly specific, probe-based assay such as TaqMan real time PCR requires high complementarity for probe binding, which might result in a failure to detect high sequence variability in the probe-binding region. Design of primers is a critical step in the development of SYBR-Green based techniques that needs to be thoroughly conducted in order to assure the necessary specificity of the test on virus detection. The tests described here were found to be highly sensitive and specific in the detection and differentiation of DWV, BQCV, and KBV. The high sensitivity of the SYBR-Green based RT-PCRs demonstrated here, which is likely to be higher than the expected for conventional



**Figure 2.** Residual risk (RU) associated with region-level proportion of samples positive to black queen cell virus (BQCV, a), to deformed wing virus (DWV, b), and to Kashmir bee virus (KBV, c) on a survey of 294 bee colonies conducted from 2004 through 2006 in continental Spain, after accounting for the number of samples collected and the number of bee colonies per region. Yellow and blue shades indicated values of  $RU \leq 1$  and  $RU > 1$ , respectively. No samples from Catalonia were obtained.

RT-PCR techniques, is important in the detection of honeybee viruses because of the silent nature of many honeybee viruses infections (Bailey et al., 1981; Evans and Hung, 2000; Hung, 2000).

The proportion of samples positive to the viral agents assessed here is consistent with estimates obtained from surveys conducted in other countries. The proportions of 0.84 (CI95%: 0.80–0.89) and of 0.68 (CI95%: 0.63–0.73) samples positive to DWV and to BQCV found here were within the range of 0.76–1.00 and 0.30–0.91 estimated respectively for each disease in other countries (Tentcheva et al., 2004; Antunez et al., 2006; Berenyi et al., 2006; Forgách et al., 2007). Similarly, the low proportion of samples positive to KBV found here (0.02; CI95%: 0.00–0.03) is consistent with estimates obtained in Uruguay, Austria, Hungary, and England (Tentcheva et al., 2004; Antunez et al., 2006; Berenyi et al., 2006; Forgách et al., 2007; Ward et al., 2007). KBV is considered the most virulent of all known honeybee viruses (Allen and Ball, 1995). High virulence is usually associated with a high transmission rate, leading to a rapid spread of the disease in an infected population. If disease transmission is sufficiently rapid, all infected bees in a beehive may potentially die or cure before they are able to establish an effective contact with susceptible bees in another beehive. Therefore, the epidemic might potentially die off due to a lack of susceptible individuals in conditions of being infected. Conversely, low virulence might result on slow, yet sustainable, between-beehive transmission rate, which could potentially lead to broad virus spread compatible with the observed for DWV and BQCV in Spain. Therefore, the low proportion of samples positive to KBV might be explained at least in part by the low analytical sensitivity of the assay for KBV detection. Note, however, that analytical sensitivity of the test estimated here suggests that the assays are reliable for routine diagnostics. Therefore, the low proportion of samples positive to KBV could alternatively be explained by low amounts of KBV RNAs in the samples or simply by a low prevalence of the disease in the country. Because samples were not randomly obtained from the

susceptible population, extrapolation of results from the subpopulation of samples analyzed here to the entire Spanish honeybee population could potentially be biased. Producers that are experiencing or that have recently experienced mortality of their colonies are more likely to submit samples for diagnosis or to accept that samples from their bee colonies are taken. Therefore, it is likely that the proportion of positive samples might be higher than the true disease prevalence in the field, at least for some of the diseases assessed here. This reason may also explain, at least in part, why the proportion of samples positive to at least one of the viruses was similar ( $P = 0.33$ ) in bee colonies with and without clinical signs of disease.

After adjusting for the number of samples collected per region, the proportion of positive samples was associated with the number of bee colonies per region, as evidenced by the value of  $RB > 1$  estimated for the three diseases assessed here. The 95%CI of  $RB$  included the value of 1 for the three diseases assessed here, and for that reason results cannot be considered significant. However, wide values for the 95%CI of  $RB$  are most likely to be related with the use of non-informative priors for the coefficients of the Bayesian regression model. Use of informative priors that assume a positive association between number of bee colonies and proportion of positive samples in a region would certainly have resulted in significant estimates for the association. However, use of non-informative priors was preferred because testing the null hypothesis of non-association between number of bee colonies and proportion of positive samples was specifically one of the objectives of the paper. Therefore, point estimates of  $RB > 1$  were assumed to represent enough evidence of positive association between number of bee colonies and proportion of positive samples in a region. In the absence of an active and formal prevention and surveillance program, bee viruses are more likely to be introduced into regions with the largest number of bee colonies, simply because larger numbers of susceptible populations are expected to be associated with higher chances for establishing effective contacts with infected individuals. An increment of 355 830 in the

number of bee colonies in a region was associated with increments of 3%, 12%, and 74% in the risk of finding, respectively, DWV, BQCV, and KBV. Considering that the range in the number of bee colonies per region in Spain is way above that figure – i.e., the difference between the maximum and minimum values of 547 728 and 12 933 bee colonies recorded, respectively, in Andalusia and Cantabria, which equals 534 795 – this finding may be useful in the development of a comprehensive surveillance and control program in the country. Because the allocation of resources is primarily regionally based in Spain, the number of bee colonies per region might represent a relatively simple initial indicator of how resources could be allocated in order to develop a preliminary national surveillance program aimed at obtaining situational awareness on the true prevalence of bee diseases in the field.

The residual risk (RU) remaining after adjusting for the number of samples collected and the number of bee colonies in a region was mapped for the three diseases assessed here (Fig. 2). Values of  $RU > 1$  for a given region and disease suggest the presence of factors or forces other than number of bee colonies in the region that are increasing the risk for the disease in the region. In other words, for those regions with values of  $RU > 1$  for a given disease, the number of bee colonies and the number of samples collected alone were not able to explain the high proportion of positive samples observed in the region. Factors or forces that were not assessed here are varied and include, for example, selective willingness of producers in certain regions to send samples that are more likely to be virus-infected, higher chances of importing infected material or individuals, or presence of environmental or epidemiological conditions that favour the spread of the disease. Visual inspection of the residual risk (Fig. 2) suggests that the presence of those factors or forces might increase eastwards and southwards, as indicated by the values of  $RU \leq 1$  estimated for many of the northern and western regions in opposition to the values of  $RU > 1$  mostly estimated in southern and eastern regions. Spatial concentration of residual risk could be associated with specific management practices

or environmental conditions in those regions found to be at a higher risk for the disease. In fact, it has been hypothesized that honeybee viruses are widespread and mostly responsible for unapparent or persistent infections. However, certain environmental factors, like climatic change or parasite infestations with agents like *Varroa destructor*, *Nosema apis*, or *Nosema ceranae* (Bailey et al., 1983; Shimanuki et al., 1994; Martín-Hernandez et al., 2007) may activate latent viral infections and, eventually, lead to the appearance of clinical symptoms (Bailey et al., 1981; Evans and Hung, 2000; Hung, 2000). One can imagine that the increase in residual risk observed in southern and eastern regions could be associated with higher prevalence or presence of one or more of these factors believed to promote active viral infection. For example, presence of *Nosema ceranae* has been demonstrated in Spain, (Martín-Hernandez et al., 2005), although little is known about the spatial distribution and relative abundance of this agent in the country. Based on previous knowledge on honeybee viral disease epidemiology and on the findings of the survey presented here, it is possible to speculate that there might be factors or forces selectively promoting the introduction, transmission, and/or spread of honeybee viral diseases on certain regions of Spain. Future research should be aimed at specifically assessing the hypothesis of spatial clustering of honeybee viral diseases in Spain and to identify and quantify the nature and extent of the association between disease prevalence and factors hypothesized to promote or prevent disease spread.

In summary, this study provides estimates of the analytical sensitivity and specificity of a real time RT-PCR for diagnostic of DWV, BQCV, and KBV in Spain. The survey demonstrated the relatively broad spread of DWV and BQCV throughout continental Spain and confirmed the presence of KBV in certain regions of the country. Evidence of the association between proportion of positive samples and number of bee colonies per region was provided. Results will be useful in the development of programs for surveillance and control of the three most important viral diseases affecting the honeybee industry of Spain.

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### Sensibilité analytique et spécificité d'une RT-PCR pour diagnostiquer et caractériser la répartition spatiale de trois maladies virales d'*Apis mellifera* en Espagne.

#### *Apis mellifera* / virose / virus ARN / risque associé / RT-PCR en temps réel

**Zusammenfassung – Analytische Sensitivität und Spezifität einer RT-PCR Methode für die Diagnose und Charakterisierung der räumlichen Verteilung von drei *Apis mellifera* Viren in Spanien.** Spanien gilt als der weltweit drittgrösste Produzent für Honigbienen (*Apis mellifera*). Demgegenüber stehen von Viren wie dem Verküppeltem Flügelvirus (DWV), dem Schwarzen Königszellenvirus (BQCV) und dem Kaschmir Bienenvirus (KBV) hervorgerufene Krankheiten, die als die wichtigsten Ursachen für virale Krankheiten der Honigbiene gelten und schwerwiegende epidemiologische und wirtschaftliche Schäden in befallenen Bienenständen verursachen können. Voraussetzung für die Kontrolle dieser viralen Infektionen ist die Entwicklung hochspezifischer und hochsensitiver Diagnosemethoden und die Kenntnis der landesweiten Verbreitung dieser Krankheiten. Für Spanien gilt es insbesondere auch, vorläufige Daten über die Auswirkungen dieser Krankheiten auf die Honigbienen produzierende Industrie zu erfassen. Hauptziel dieser Arbeit war es, die analytische Sensitivität und Spezifität von RT-PCR-Methoden für die Identifizierung von DWV-, BQCV- und KBV-Infektionen in spanischen Bienenvölkern zu ermitteln. Insgesamt wurden zwischen den Jahren 2004 und 2006 Proben aus 294 Bienenvölkern der 14 von 15 Verwaltungsregionen Spaniens (ohne Inselregionen) gesammelt (Abb. 1). Für die Detektion von DWV und BQCV nutzten wir eine auf SYBR-Green-Inkorporation beruhende Einschritt-Realtime RT-PCR-Methode (Kukielka et al., 2008) und für die KBV-Detektion modifizierten wir eine konventionelle RT-PCR-Methode (Stoltz et al., 1995) für Realtime-Bedingungen. Die analytische Spezifität der RT-PCR-Methoden wurde durch Sequenzanalyse der Amplifikations-

produkte überprüft. Die Sensitivität der Realtime RT-PCR-Ansätze lag bei  $10^{-9}$  Viruspartikel für DWV und BQCV und bei  $10^{-5}$  für KBV. Zur Analyse der Verbindung zwischen Virusvorkommen und Anzahl und räumlicher Verteilung der Bienenvölker nutzten wir ein auf einem Bayes'schen Verfahren beruhendes Modell. Dabei zeigte sich, dass DBV mit 84,0 % das am häufigsten vorkommende Virus in den Stockproben war, gefolgt von BQCV (68,0 %) und KBV (1,7 %). Eine Ko-Infektion durch zwei Viren, meist mit BQCV und DWV, wurde in 60,4 % der Proben gefunden. Die visuelle Begutachtung des krankheitsspezifischen Restrisikos lässt erwarten, dass hier nicht erfasste Faktoren einen Anstieg des Risiko für einen DWV-, BQCV- und KBV-Befall in Spanien in östlicher und südlicher Richtung mit sich bringen können (Abb. 2).

Der Anteil der in dieser Studie gefundenen positiven Probe für pathogene Bienenviren entspricht dem von Schätzwerten aus anderen Ländern. Der Anteil positiver Proben war auch assoziiert mit der Anzahl an Bienenvölkern in der jeweiligen Region. In Abwesenheit aktiver und formaler Präventions- und Überwachungsprogramme ist deshalb zu erwarten, dass Bienenviren vor allem in Regionen mit der grössten Bienenichte eingeführt werden und sich dort aufgrund der erhöhten Kontaktwahrscheinlichkeiten mit infizierten Bienen verbreiten können. Die Ergebnisse dieser Studie sollten von Nützlichkeit sein für die Entwicklung von Überwachungs- und Kontrollprogrammen für Viruskrankheiten, die die Honigbienenindustrie Spaniens treffen können.

#### Realtime RT-PCR / *Apis mellifera* / RNA Viren / Assoziiertes Risiko

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