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Short communication. First report of black queen-cell virus detection in honey bees (*Apis mellifera*) in Spain

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Abstract

The black queen-cell virus (BQCV) is a RNA virus recently classified within the Family *Dicistroviridae,* genus *Cripavirus*. Although BQCV is found worldwide, it has never been previously reported in Spain in spite of the fact that this country is the main producer of honey bees (*Apis mellifera*) in the European Union. This study presents a clinical and laboratory description of a BQCV outbreak in honey bees within an apiary made up of 80 colonies in the province of Toledo (Spain). Mortality was associated with signs of diarrhoea, enlarged abdomen, oedema in the midgut, and distended rectum filled with a transparent liquid. The parasite *Nosema apis* has been reported in BQCV outbreaks. The acute clinical picture, with symptoms of diarrhoea and massive deaths of adult bees, could be related to the association between *N. apis* and the BQCV since, under normal circumstances, cases of nosemosis caused by *N. apis* reported in Spain over the last 10 years were always anecdotal and in chronic form, with no associated clinical symptoms whatsoever detected in the beehives. On the other hand, in cases of nosemosis linked to *N. ceranae*, the clinical symptoms are entirely different, with no evidence at all of diarrhoea and massive deaths around the beehive but only a progressive decrease in the population. The sample of adult bees was analysed by the polymerase chain reaction method for BQCV and a fragment of 700 bp sequenced (Acc. No. DQ132875). It was also noted that this sample had a high spore count of *N. apis* epidemiologically related to BQCV. The possible role of BQCV in the mortality and associated signs is discussed in this work.

Additional key words: beehive mortality, gross pathology, infectious diseases, *Nosema apis*, RT-PCR.

Resumen

Comunicación corta. Primera descripción en España de la enfermedad del virus de la realera negra en abejas melíferas (*Apis mellifera*)

El virus de la realera negra (BQCV) es un virus RNA que ha sido recientemente clasificado dentro de la familia *Dicistroviridae,* género Cripavirus. Aunque BQCV se halla distribuido mundialmente, nunca había sido descrito con anterioridad en España, a pesar de ser este país el principal productor de abejas melíferas en la Unión Europea. En el presente estudio, se realiza una descripción clínica y laboratorial del brote de BQCV en un colmenar profesional compuesto por 80 colmenas ubicado en Toledo (España). Se observó mortalidad asociada a signos de diarrea, abdomen aumentado, edema en el ventrículo, y distensión del abdomen, relleno con líquido transparente. Este cuadro clínico agudo probablemente es debido a una potenciación de la acción patógena de *Nosema apis* por el citado virus, dado que en España, en los últimos 10 años, la nosemosis debida a *N. apis* se manifiesta exclusivamente de forma crónica, sin síntomas aparentes y sin mortalidad o debilidad asociada en las colmenas, a diferencia de la nosemosis debida a *N. ceranae* que provoca despoblamiento de las colmenas y muerte en un periodo variable de tiempo. La muestra de abejas adultas fue positiva a BQCV, mediante la reacción en cadena de la polimerasa, y se secuenció un fragmento de 700 pb (N.º acc. DQ132875). Esta muestra también poseía un elevado recuento de esporos de *N. apis*, microsporidio relacionado epidemiológicamente con BQCV. Se discute en el presente trabajo el posible papel de BQCV en la mortalidad y en los signos asociados.

Palabras clave adicionales: enfermedades infecciosas, lesiones macroscópicas, mortalidad de colonias, *Nosema apis,* RT-PCR.

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The black queen-cell virus (BQCV), a RNA virus previously part of the «picorna-like» group of viruses affecting honeybees, has recently been classified within the new genus Cripavirus (Family *Dicistroviridae*) (Mayo, 2002). The name of the virus was derived from darkened areas on the walls of the cells containing infected pupae (Benjeddou *et al.,* 2002). Excluding the poly-A tail, its genome has 8,550 nt, with two open reading frames (ORF). The 5' proximal ORF encodes a putative replication protein, while the 3' proximal ORF encodes a capsid polyprotein (Leat *et al.,* 2000).

BQCV is a common virus that affects honeybees; however its implication in honeybee mortality is currently poorly understood. While it is known that BQCV is the major cause of queen larvae mortality in Australia (Anderson, 1993), the virus has been detected in 86% of adult samples and 23% of pupae on a survey in healthy French bee colonies (Tentcheva *et al.*, 2004), and recently in Austrian apiaries (Berényi *et al.,* 2006). It seems that the parasite *Nosema apis* could be implicated in the pathogenesis of BQCV outbreaks (Bailey *et al.,* 1983; Allen and Ball, 1996), but no exhaustive study is available on the matter. *Nosema apis* is a microsporidia parasite of the honey bee that invades the epithelial cells of the midgut of adult bees (Bailey, 1955). It is considered the main transmission route of BQCV (Bailey *et al.,* 1983). In its acute form, it causes bee dysentery leading to diarrhoeal faeces, shortens the life span of the individuals and causes greater colony mortality (Fries *et al.,* 1984).

Although there is an important concern worldwide about viruses that affect honey bees, as reflected in the abundant and recent bibliography, there is not enough data regarding the occurrence of these viruses in Spain, in spite of the fact that it is the main producer of honey bees in the European Union. The present study represents the first description of BQCV disease in Spain.

Bees with pathological symptoms (enlarged abdomen and jerky movements) were obtained from an apiary made up of 80 colonies from Toledo (Central Spain) in which an important mortality of bees at the entrance of the beehives had been observed. The veterinary in charge of the apiary collected the samples by sweeping the entrance to the beehive. They were placed in cardboard boxes available in the Centre (Centro Apícola Regional, Guadalajara, Spain) and specially designed for this purpose. Although the shipment was sent to the Centre by courier service, the bees were moribund when they arrived. One hundred of them were used to conduct parasitology tests, and the remainders (about 50 bees) were frozen at –80ºC awaiting the virological analysis. The main clinical feature in all the colonies was the presence of signs of diarrhoea and bee death. The gross pathology of the necropsied bees revealed an enlargement of the abdomen, oedema in the midgut with a pallid aspect, and a distended rectum filled out with a transparent liquid.

In order to establish a complete diagnostic, laboratory tests were carried out. The standardized methods described by the OIE (2004), to determine the presence of *Varroa destructor*, *Acarapis woodi* and *Malpighamoeba mellificae* were followed. The determination of *Nosema* sp. was carried out by the polymerase chain reaction (PCR) previously described (Higes *et al.,* 2006).

RT-PCR of BQCV was conducted on the frozen adult samples. A negative control of bees was obtained from a healthy bee colony from Guadalajara (Central Spain). Sample preparation prior to RNA extraction was carried out macerating 10 bees with 5 ml of phosphate buffer saline (PBS). After centrifugation at 690 g for 15 min, the supernatant was collected. RNA extraction was carried out on 100 µl of macerated sample using the TriPure Reagent (Roche Diagnostics) method, following the manufacturer's instructions. One step RT-PCR was performed based on the protocol previously described (Benjeddou *et al.,* 2001), which amplifies a 700 bp region, corresponding to nucleotides 7,850 to 8,550, at the 3' end of the genome. Purification of DNA fragment of 700 bp was realized prior to sequencing, using GFX PCR DNA and gel band purification kit (Amersham Biosciences), following the manufacturer's instructions. The sequencing reaction was realized in an ABIPrism 3100 sequencer (Applied Biosystems). Sequenced products were compared to those described in the Genbank using the Blast search.

Bees with viral symptoms obtained from Toledo were positive to *N. apis*, in which a mean of 11.85×10^6 spores per bee were counted, while it was negative for *V. destructor*, *A. woodi* and *M. mellificae*. The lesions observed were compatible with cases of acute nosemosis induced by *N. apis*. The sample was also RT-PCR positive for the BQCV, showing the expected band of 700 bp (Fig. 1). In contrast, the healthy bees from Guadalajara were negative to *Nosema* sp. and BQCV. The results of sequencing confirmed a novel sequence of BQCV that was incorporated to the GenBank (Acc. No. DQ 132875).

Presence of diarrhoea at the entrance of the beehive is a characteristic feature of acute nosemosis (OIE,

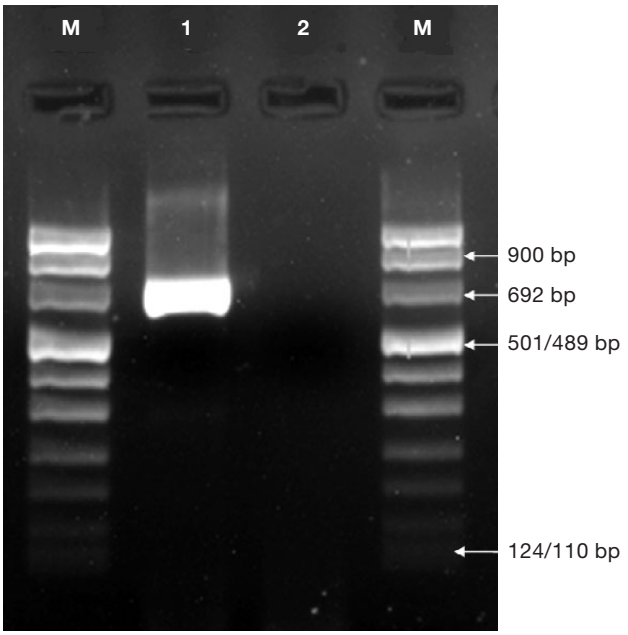

Figure 1. RT-PCR from a 700 bp section of Black queen-cell virus RNA. M: molecular weight marker VIII (Roche-Applied Science). Lane 1: positive sample (HH 285). Lane 2: negative control sample (HH 286).

2004). In this study signs of diarrhoea have been observed in bees with small levels of spores of *Nosema apis* (11.85×10^6 spores per bee). Nevertheless, there is not consensus concerning the microsporidian load necessary to cause acute disease (OIE, 2004). Furthermore, higher spore levels of *N. apis* (i.e. 46×10^6 spores per bee or more) have been also observed in colonies of other Spanish regions that were negative for BQCV and in which symptoms of diarrhoea or bees death were absent (data not shown). Similar results have also been described in France in colonies where *N. apis* was found but no clinical features were observed (Chauzat *et al.*, 2006). Recently Higes *et al.* (2007) asserted that *N. apis* is less pathogenic that *N. ceranae* to *Apis mellifera iberiensis*. In Spain and other European countries, acute symptoms induced by *N. apis* have not been observed in the last 10 years although this microsporide is commonly found in colonies of asymptomatic bees (Martín-Hernández *et al.,* 2005; Chauzat *et al.,* 2006). For this reason, the increase in clinical features observed could be associated with the BQCV in the affected colonies. The possibility that a coinfection between *N. apis* and BQCV could weigh down the clinical course should be taken into account. At the present, there is no data available about clinical manifestations in adult bees coinfected by BQCV and *N. apis*. However, it has been suggested previously that this organism may be implicated in mortality due to *N. apis* (Benjeddou *et al.,* 2001). In addition, it seems that bee mortality could be the result of a complex interaction among multiple factors in which viruses and parasites are involved (Hung *et al.,* 1996; Martin, 2001; Benjeddou *et al.,* 2002). In the future, it will be necessary to establish the relationships between all the factors implicated in bee mortality. Therefore, pathological and epidemiological studies will be conducted. In conclusion, the main finding of this work was the first isolation of BQCV in Spain and the possible role of this virus to increase de pathogenicity of the honey bee parasite *N. apis*.

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