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Original Article

First molecular detection of Chronic Bee Paralysis Virus (CBPV) in Iran

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ABSTRACT

Among the viruses infecting honey bees, chronic bee paralysis virus (CBPV) is known to induce significant losses in honey bee colonies. CBPV is an unclassified polymorphic single stranded RNA virus. Using RT-PCR, the virus infections in honey bees can be detected in a rapid and accurate manner. Bee samples were collected from 23 provinces of Iran, between July-September 2011 and 2012. A total of 160 apiaries were sampled and submitted for virus screening. RNA extraction and RT-PCR were performed with QIAGEN kits. The primers lead to a fragment of 315 bp. The PCR products were electrophoresed in a 1.2 % agarose gel. Following the RT-PCR reaction with the specific primers, out of the 160 apiaries examined, 12 (7.5 %) were infected with CBPV. This is the first study of CBPV detection in Iranian apiaries. We identified CBPV in the collected samples from different geographic regions of Iran.

Keywords: CBPV, Honey bee, RT-PCR

INTRODUCTION

The European honeybee, *Apis mellifera*, is found throughout the world and, as the principal pollinator of commercially important food crops, plays an important role in the global economy (Chen & Siede 2007, Delaplane & Mayer 2000). The scientific interest in viral diseases of the honeybee has been increasing considerably during the past few years (Allen & Ball 1996). Among the viruses infecting honey bees, chronic bee paralysis virus (CBPV) is the causal agent of chronic paralysis; known to induce significant losses in honey bee colonies (Ball & Bailey 1997). Serological and infectivity tests showed that chronic bee-paralysis virus was concentrated in the brains of chronically paralyzed bees. Electron microscopy of the brains showed particles resembling chronic bee-paralysis virus but these may have been synaptic vesicles or sectioned microtubules, since similar particles were also seen in the brains of apparently healthy bees. These particles also resembled particles that were seen in sections of pellets of purified chronic bee-paralysis virus, and that were electron-transparent in the centre (Bailey & Milne 1969). A correlation between chronic paralysis and high viral loads of CBPV was demonstrated particularly in symptomatic bees. Moderate viral loads were also demonstrated in colonies without symptoms (Blanchard *et al* 2007). CBPV is transmitted through food or wounds. Two manifestations of the infection have been noted, as

follows. (I) The bees are unable to fly, they tremble and crawl, and the wings are asymmetrically outspread. They often suffer from dysentery and die within a few days. (II) The bees look black because of hair loss. The guard bees do not recognize them and dismiss them because of their altered look. Although in some cases up to 30% of worker bees are affected, CBPV infection sometimes remains undetected, and the colonies usually recover spontaneously from the disease (Bailey 1967). Chronic paralysis virus has a world-wide distribution (Allen & Ball 1996). There is no evidence in the literature that strains of the virus vary in pathogenicity. Susceptibility to bee paralysis has been shown to be linked to hereditary factors (Kulincevic & Rothenbuhler 1975), and the prevalence of paralysis is usually quite low (Bailey & Ball 1991). Current diagnosis of the clinical disease is based on an Agarose Gel Immuno Diffusion (AGID) test (Ball 1999, Ribière *et al* 2000), complemented by RT-PCR (Ribière *et al* 2002). First isolated in 1963 (Bailey *et al* 1963), the aetiological agent of this pathogen is the Chronic bee paralysis virus (CBPV), an unclassified polymorphic (particles 20 x 30 to 60 nm) single stranded RNA virus (van Regenmortel *et al* 2000). Recent advances in molecular technology have greatly expanded our ability to detect and elucidate the molecular events associated with virus infections and pathogenesis (Ghosh *et al* 1999, Olivier *et al* 2008). Using RT-PCR based assays, the virus infections in honey bees can be detected and quantified in a rapid and accurate manner (Chen *et al* 2006a, Chen 2006b). Our objective was to evaluate the presence of chronic paralysis virus in honey bee colonies in Iran.

MATERIALS AND METHODS

Sampling of bees. Infected honeybees from colonies suffering with symptoms of depopulation, sudden collapse, paralysis, and varroa infestation, or colonies that already have these symptoms but now they are apparently healthy, used in this study, originated from 23 provinces of Iran (various geographic regions): Alborz, Ardebil, Chaharmahal and Bakhtiari, West Azarbayejan, East Azarbayejan, Fars, Ilam, Isfahan, Ghazvin, Gilan, Golestan, Hamedan, Kerman, Kermanshah, South Khorasan, Khorasan Razavi, North Khorasan, Kohkilouyeh and Boierahmad, Mazandaran, Markazi, Semnan, Yazd, Zanjan). Bee samples were collected between July - September 2011 and 2012 and sent to Honey Bee Department of Razi Vaccine and Serum Research Institute, by collaborating colleagues in Veterinary Organization. Altogether, samples from 160 apiaries were collected and submitted for virus screening. From each apiary, 100-500 adult worker bees were sampled. All samples were transported by airplane or express mail in carefully wrapped paper sacks or boxes using cold chain and stored at -20 °C.

Isolation of RNA. The bees were homogenized in ceramic mortars with sterile diethylpyrocarbonatetreated water. The homogenates were centrifuged at 2000g for 1 min, and 140 µl of supernatant used for RNA extraction (Bere´nyi *et al* 2006). RNA was extracted employing the QIAmp Viral RNA Mini kit (QIAGEN, Germany) according to the manufacturer's instructions.

RT-PCR. Positive control was prepared from Feredrich-Loeffler-Institute in Germany. RT and amplifications were performed with a RT-PCR method by employing a One Step RT-PCR kit (QIAGEN, Germany), following the manufacturer's recommendations. For detecting CBPV infection in the honey bee, CBPV- specific primers (F: TGTCGAA CTGAGGATCTTAC and R: GACCTGATTAACGA CGTTAG) that lead to a fragment of 315 bp were used according to a previous work by Bere´nyi et al. (2006). Amplifications were performed in a Master Cycler Gradient Eppendorf. The reverse transcription at 50 °C for 30 minutes was followed by a denaturation and polymerase activation step at 95 °C for 15 min and by 40 cycles of PCR, each consisting of 30 s at 94 °C, 50 s at 55 °C, and 1 minutes at 72 °C steps. Reactions were completed by a final elongation step for 7 minutes at 72 °C (Bere´nyi *et al* 2006). The PCR products were electrophoresed in a 1.2 % agarose gel and stained with ethidium bromide. Bands were photographed under UV light with Panasonic Digital Camera.

RESULTS

A total of 160 honeybee samples originated from 23 Iranian provinces, were investigated by RT-PCR for the presence of the CBPV, one of the important honeybee viruses. Following the RT-PCR reaction with the specific primers on the isolated RNA, an approximately 315 bp product was detected (Figure). Out of the 160 apiaries examined, 12 (7.5 %) were infected with CBPV (Table). Positive samples were sent for sequencing, the results, confirmed CBPV too.

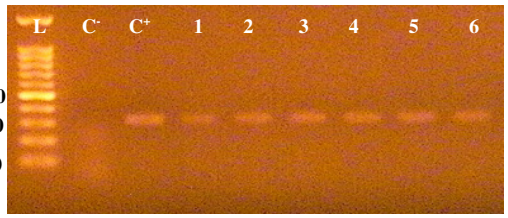

Figure. Visualization of the RT-PCR products obtained with amplifying RT-PCR fragments of 315 bp. (6 out of 12 samples were showed).L: Ladder 100 bp, C^- : Negative control, $C^{+:}$ Positive control, 1-6**:** Positive Samples

Table. Positive samples from apiaries during summer 2011- 2012.

Province	Number of apiary	Positive Apiary	Positive Province
23	160	12 (7.5 %)	6 (26 %)

DISCUSSION

A large variety of viruses multiply in the honey bee *Apis mellifera* L. (Allen & Ball 1996). Knowledge of the spreading mechanism of honey bee pathogens within the hive and apiary is essential to our understanding of bee disease dynamics. In this study, we checked out CBPV in bee samples collected from 160 infected colonies of selected Iranian apiaries in the spring and summer 2011-2012. The samples originated from different geographic areas in Iran, were sent to our Institute for causal diagnosis of health problems in affected colonies, in which the symptoms could not be explained by mere parasite infestation, bacterial or fungal infections, or noninfectious reasons. Therefore, viral infections were presumed to contribute significantly to the observed diseases. In our opinion, analysis of problematic colonies gives a more informative picture of the real pathological impact of viruses, although by this approach the sample collection cannot be planned beforehand, and the number of samples may in some cases be not high enough for statistical analysis. Because relatively small numbers of samples were received from provinces, only the occurrence of the studied virus is mentioned, but not their prevalence. In our research, to screen for virus infection in honey bee colonies, RNA samples isolated from adult bees were tested for the presence of CBPV by the RT-PCR. If bees collected from the colonies had detectable virus, the virus status of the colonies was defined as mono-infection. Besides a practical approach for the application of RTPCR- based diagnostic methods on affected colonies of problematic apiaries, we attempted to provide data for the estimation of a connection between the presence of viruses and manifest diseases of bee colonies too. Only 12 (7.5 %) CBPV-positive samples was found among the collected samples. In France, CBPV has been detected by serology in extracts of dead adult *Apis mellifera,* thus confirming that overt disease can be present at any time of the year (Faucon *et al* 2002, Faucon *et al* unpublished data). However, the highest rates of bee mortality occurred in spring and summer (Giauffret *et al* 1967, Ribiere *et al* 2002, Ribiere unpublished data). Tentcheva et al (2004) also reported sporadic detection of CBPV over the year in adult bees sampled from 360 apparently healthy colonies in different regions of France. The virus was always detected at low frequencies (in at most 4% of colonies during summer). Despite these low frequencies, CBPV was found in adult bees in 28% of the 36 surveyed apiaries (Tentcheva *et al* 2004). The virus has recently been detected by molecular techniques in honey bee samples collected during December in Uruguay. The presence of CBPV was apparently associated with episodes of bee mortality but without the trembling and crawling symptoms typical of paralysis outbreaks (Antunez *et al* 2005). In the mid 1990's, CBPV was detected as a cause of unusual and severe adult bee mortality in the Canary Islands. More than 75% of samples of dead adult bees collected in spring and early

summer contained large amounts of virus, sufficient to be detected serologically in immunodiffusion tests. The increased prevalence of CBPV in these islands may have been associated with high colony densities or possibly with increased susceptibility to infection due to inbreeding (Ball 1999). Tentcheva and colleagues did not detect CBPV in samples of pupae collected in apparently healthy colonies from 360 hives in France, during spring, summer and autumn of 2002. However the virus was detected in 4% of adult bees during the summer (Tentcheva *et al* 2004). Infectivity tests and laboratory experiments to investigate the incidence and prevalence of CBPV in Britain indicated that the virus was endemic in many apparently healthy colonies with no regular seasonal cycle of occurrence (Bailey 1967). At present it is possible with molecular diagnosis to detect inapparent CBPV infections in individuals without the need for complex and time consuming infectivity tests (Ribiere *et al* 2002, Tentcheva *et al* 2004).

This is the first project of CBPV detection in Iranian apiaries. We identified CBPV from different geographic regions of Iran. Trade and exchange of infected animals, contaminated equipment, and bee products between apiaries, regions, or even countries may be of greater importance in the spread of viruses. Therefore, virological and parasitological investigations should be considered before import and export of bees and bee products.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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