Focus on a laboratory

Detection of main honey bee pathogens by multiplex PCR
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The widespread decline of the global bee population in recent years, a result of unexplained bee colony collapses and declines, highlights the potential involvement of a variety of fungal, bacterial and viral infections. These infections, which are not systematically diagnosed, lead to uncertainty about the causes of the phenomenon. The aim of the project was to provide tools for the rapid detection of the major honey bee pathogens that are potentially involved. This was achieved by the development of multiplex PCR for the detection of 14 pathogens (seven viruses, four bacteria, three fungi), as well as the development of quantification tools.

Table 1. Viral, bacterial and fungal honey bee pathogens covered by the Multi-Path project

<table>
<thead>
<tr>
<th>Viral agents (RNA virus)</th>
<th>Bacterial agents</th>
<th>Fungal agents</th>
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<tr>
<td>ABPV, Acute bee paralysis virus</td>
<td><em>Paenibacillus larvae</em> (causative agent of American foulbrood)</td>
<td><em>Ascosphaera apis</em> (causative agent of chalk brood)</td>
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<td>BQCV, Black queen cell virus</td>
<td><em>Melissococcus plutonius</em> (primary agent of European foulbrood)</td>
<td><em>Nosema apis</em> (causative agent of nosema disease)</td>
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<td>CBPV, Chronic bee paralysis virus</td>
<td><em>Paenibacillus alvei</em> (associated or secondary agent of European foulbrood)</td>
<td><em>Nosema ceranae</em></td>
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<td>DWV, Deformed wing virus</td>
<td><em>Enterococcus faecalis</em> (associated or secondary agent of European foulbrood)</td>
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<td>IAPV, Israeli acute paralysis virus</td>
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<td>KBV, Kashmir bee virus</td>
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<td>SBV, Sacbrood virus</td>
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* The two secondary agents of European foulbrood are not included in the three Multiplex PCRs targeting the main bee micro-pathogens, but are identified by a supplementary Multiplex PCR.

(1) AFSSA and AFSSET merged in July 2010 to create ANSES, (French Agency for Food, Environmental and Occupational Health & Safety).
12 major micro-pathogens, as well as a fourth multiplex PCR reserved for the detection of the causative bacterial agents of foulbrood (Table 1). The three main multiplex PCRs comprise two PCRs dedicated specifically to the detection of the seven bee RNA viruses, thus ensuring more reliable detection in the event of genetic variability, and a third PCR for the detection of pathogens with DNA genomes, i.e. the two main causative bacterial agents of American and European foulbrood, and three fungi. In addition to the two main causative bacterial agents of foulbrood, the fourth multiplex PCR also simultaneously detects the two secondary bacterial agents of European foulbrood (Paenibacillus alvei and Enterococcus faecalis). To do this, a preliminary inventory was drawn up of the different primers that have been published and are specific to each pathogen. For pathogenic agents that did not have a published PCR or whose amplification sizes were incompatible with the detection of other pathogens, specific primers were determined using molecular computing tools and the sequencing of different genomes.

These tools were tested and validated on adult bee and brood samples that had previously been characterised in the laboratory (see Figure 1 for an illustration of two multiplex PCRs). Moreover, these tools were tested on almost 400 bee samples collected in France between 2008 and 2009 from both affected apiaries and those with no apparent problems. The three multiplex PCRs were also tested on royal jelly and potential vectors of micro-pathogens such as bee macro-pathogens (Varroa destructor and Tropilaelaps clareae) mites, the Aethina tumida small hive beetle, and the Vespa velutina Asian hornet), as well as on other insects such as ants and wasps found frequently in the bee environment. The results of this last sample category showed the presence of several viruses, which indicates that these tools could be used to better characterise the distribution of these pathogens in different insect populations.

In addition to this research, adjustments to multiplex PCRs targeting pathogens that induce closely related or similar symptoms are being developed or finalised. That is the purpose of the fourth multiplex PCR developed at the laboratory for the simultaneous detection of American and European foulbrood bacterial agents. These two brood diseases, which can develop in a colony at the same time, share symptoms such as a mosaic brood pattern, light yellow to brown dead larvae and foulbrood scales. During its development, the tool’s repeatability and analytical sensitivity were established by determining the detection threshold based on a set of reference plasmids finalised at the laboratory. Similarly, a multiplex PCR will be developed to detect the causative agents of larval diseases (SBV, BQCV and Ascosphaera apis), as well as a protocol enabling the simultaneous detection of CBPV, Nosema apis and N. ceranae and of the causative agent of acarine disease (Acarapis woodi), which can be used for field bee deaths in season.

All of this research provided an opportunity to acquire sound experience in the development of multi-pathogen detection tools and then put that experience into practice, particularly for the PCR differentiating between N. apis and N. ceranae during the diagnosis of nosema disease. In France, only N. apis is considered to be a causative agent of nosema disease, an illness that affects adult bees and results in depopulation, a weakened colony and high colony mortality. After obtaining spore counts by light microscopy, the Nosema spp. species is identified by multiplex PCR. This PCR is based on the detection of a 16S rRNA sequence of the parasite specific to each of the two species (N. apis and N. ceranae). The protocol published by Martin-Hernandez et al. (2007) and reproduced in the OIE Manual (OIE, 2008) was adapted and its sensitivity improved in the laboratory. The initial protocol had been modified to enable the detection of both of the species, including when there is more of one species than the other. Accurately differentiating these two pathogens has major implications for epidemiology and our understanding of the disease.

In addition to these qualitative tools, quantification tools for the six viruses ABPV, BQCV, DWV, IAPV, KBV and SBV, not yet available at the laboratory, were developed by quantitative PCR (2). The protocols for quantitative PCRs already published for the quantification of the main agents of American (Han et al. 2008) and European (Roetschi et al. 2008) foulbrood were also adapted.

This research provided the laboratory with multi-pathogen molecular diagnostic assays for the detection or rapid identification of all of the major pathogens in bee or brood samples. These tools can be used in particular during...
occurrences of bee colony mortality or decline where there are no characteristic symptoms indicating a given disease. They are complemented by quantitative molecular tools that can more accurately define the degree of involvement of a pathogen during these events.

References


(2) A quantitative PCR targeting CBPV had previously been developed in 2007 (Blanchard et al. 2007).