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Recent molecular biology methods for foulbrood and nosemosis diagnosis

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Summary
Honey-bee colony losses are an increasing problem in Western countries. There are many different causes, including infections due to various pathogens. Molecular biology techniques have been developed to reliably detect and identify honey-bee pathogens. The most sensitive, specific and reliable is the quantitative real-time polymerase chain reaction (qPCR) methodology. This review of the literature describes various studies where qPCR was used to detect, identify and quantify four major honey-bee pathogens: the bacteria *Paenibacillus larvae* and *Melissococcus plutonius* (the causative agents of American foulbrood and European foulbrood, respectively) and the microsporidia *Nosema apis* and *N. ceranae* (the causative agents of nosemosis). The application of qPCR to honey-bee pathogens is very recent, and techniques are expected to improve rapidly, leading to potential new prospects for diagnosis and control. Thus, qPCR techniques could shortly become a powerful tool for investigating pathogenic infections and increasing our understanding of colony losses.

Keywords
American foulbrood – Bee – European foulbrood – Honey bee – *Melissococcus plutonius* – *Nosema apis* – *Nosema ceranae* – Nosemosis – *Paenibacillus larvae* – Quantitative polymerase chain reaction.

Introduction

Honey-bee health is of major importance as it is linked to economic, agricultural, agronomic and environmental interests. However, honey bees – like other animals – are the target of numerous pathogens and parasites. The development of world trade and ease of travel have fostered the movement of diseases between continents. New diseases have been observed in Western countries, in addition to colony collapse disorder, a recent syndrome that beekeepers and scientists are finding particularly difficult to understand and combat. Numerous studies point out that colony weakening and disease development often result from the combined effects of factors such as pathogenic infections, environmental conditions or exposure to pesticides. This multifactorial aspect makes honey-bee health analysis difficult to perform. It is therefore essential to improve current diagnostic techniques, including methods to detect, identify and quantify pathogens.

Of the major diseases known to affect honey-bee colonies, two are bacterial, affecting honey-bee brood – American foulbrood (AFB) and European foulbrood (EFB) – and one is fungal, affecting adult bees – nosemosis. American foulbrood is regulated in 22 European countries, EFB in 13 and nosemosis in eight European countries (personal communication, European Union Reference Laboratory for Honey Bee Health, 2012). These diseases were previously diagnosed by combining the observation of clinical signs in colonies with microscopic analyses. Although observations often suffice for a reliable diagnosis, it appears that EFB clinical signs can sometimes be confused with those of AFB. Moreover, *Nosema apis* has been known for years to be the causative agent of nosemosis, but recently another microsporidian was found in *Apis mellifera*. *Nosema ceranae* infection does not induce any of the overt clinical signs observed after *N. apis* infection, such as diarrhoea (1). Optical microscopic analyses, which are routinely performed to identify and quantify *Nosema* spores for diagnosis, cannot discriminate between these species as the spores from both

are very similar. Molecular techniques have thus been developed to support traditional diagnostic methods.

Molecular techniques provide new and efficient tools to estimate honey-bee health. Characterised by high sensitivity and specificity, polymerase chain reaction (PCR) methods allow fast detection of pathogens and accurate identification of species. Several PCR-based diagnostic methods are recommended in the World Organisation for Animal Health (OIE) *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (the *Terrestrial Manual*) (2). However, conventional PCR methods present some technical limitations. For example, they do not allow quantification, which is important to estimate pathogen load. Quantitative real-time PCR (qPCR) methods, with better performances, have been developed to detect honey-bee pathogens. The qPCR method was first used to detect a viral honey-bee pathogen in 2005 (3).

This literature review focuses on describing the qPCR methods developed to detect four major honey-bee pathogens: the bacteria *Paenibacillus larvae* and *Melissococcus plutonius*, the causative agents of AFB and EFB, respectively; and the microsporidia *N. apis* and *N. ceranae*. The advantages and disadvantages of qPCR are also presented, along with new prospects.

Conventional polymerase chain reaction in honey-bee pathogen detection

Most molecular diagnostic methods for foulbrood and nosemosis are based on conventional PCR. This method is more sensitive and specific than microscopy, so a pathogen may be detected in the colony before the appearance of clinical signs. The *Terrestrial Manual* recommends several PCR methods for identifying bee pathogens such as *P. larvae*, *M. plutonius*, *N. apis* and *N. ceranae*. All of these methods are widely used worldwide and are considered reference methods.

Despite numerous advantages, conventional PCR also has some disadvantages and technical limits. The main limit is that true quantification of the pathogens is not achievable because:

– the conventional PCR results are obtained at the final phase of the amplification, not in the linear phase, as for qPCR analysis

– PCR products are visualised in agarose gel.

The qPCR methods can detect a change as small as two-fold, compared to agarose gel, which is able to detect about a ten-fold change. Another limit is that conventional PCR methods require a time-consuming, post-processing step, which entails the manipulation of intercalating agents for DNA staining. The most commonly used dye is ethidium bromide, which is highly hazardous for both human and environmental health. However, several developments have contributed to improvements in the methodology. In particular, new protocols have been designed, using either nested PCRs to increase the sensitivity and specificity of detection or multiplex PCRs to increase the number of target pathogens detected in the same reaction. The method recommended by the OIE *Terrestrial Manual* for discriminating between *N. apis* and *N. ceranae* is a multiplex PCR allowing the specific amplification of each microsporidian without any cross-reaction between primer pairs (4). Multiplex PCR has the further advantage of being able to simultaneously detect a wide variety of pathogens, such as viruses, bacteria and fungi (5). However, the limit of detection (LOD) is usually higher than the LOD of uniplex PCRs (6). This implies that multiplex PCR analysis may fail to detect all of the pathogens present in a given sample. A good illustration of the need to estimate the performance of PCR techniques is given by the comparative evaluation of nine published primer pairs, designed to detect and identify three *Nosema* species by conventional PCR (7). The specificity and sensitivity of the primer pairs were assessed by qPCR experiments. Results showed that all primer pairs could detect between the equivalent of 10^4 spores down to the equivalent of ten spores. However, it should be remembered that qPCR is a method that, when optimised, can potentially detect one gene copy and that the *Nosema* genome could contain multiple copies of the target gene, ribosomal RNA (rRNA) (see 8).

Quantitative real-time polymerase chain reaction in honey-bee pathogen detection

Thus, qPCR is increasingly being used in laboratories conducting molecular biology analyses, since it may resolve issues raised by conventional PCR and could offer better performance in terms of sensitivity, specificity and reliability. This method may also be simpler and safer, since the post-PCR processing step with ethidium bromide is no longer required. It may also be faster, thanks to the development of Taq polymerases that better anchor themselves to the target DNA. New PCR machines with improved performances allowing fast temperature changes can also be used. Thus, a ten-minute qPCR was described for *P. larvae* detection (9). Moreover, it was calculated that the qPCR LOD for *P. larvae*

in honey is 100 times lower than that of a culture method (10). Several studies reported detection with fewer than ten copies of the target gene. This level of sensitivity is difficult to attain with conventional PCR. Specificity is also much higher with the use of specific probes besides primers. Furthermore, software programs developed for qPCR are increasingly efficient and often offer a greater range of functionalities than software programs developed for conventional PCR. More reliable results are also obtained, since the data are collected in the exponential phase of amplification. Finally, quantification is accurate.

The qPCR methods have few disadvantages, though care should be taken when interpreting their results because of their extremely high sensitivity. Detecting small amounts of a pathogen in the colony does not mean that the disease will develop. The higher sensitivity of qPCR compared to conventional PCR (e.g. 11, 12) could also induce more frequent contamination during analytical steps. It is important to stress that almost all qPCR methods developed to detect bacterial and microsporidian pathogens on honey bees are based on DNA. This implies that non-infectious forms can also be detected. Another drawback of qPCR is its cost. This technology requires sophisticated and expensive equipment, which impedes its implementation in all laboratories in the short term. However, the absence of post-PCR steps and the possibility of easily processing a large number of samples in one run both represent substantial savings of time and money in the long term.

Quantitative real-time polymerase chain reaction and foulbrood

All the studies describing the use of qPCR for detecting *P. larvae* or *M. plutonius* were designed to exploit the major difference between conventional PCR and qPCR; namely, quantification. The qPCR methods allow researchers to reach a goal unattainable with conventional PCR, i.e. to determine the pathogen load in a given matrix which is expressed in numbers of spores or gene copies. The absolute quantification strategy consists of developing a standard curve and correlating the number of amplification cycles determined by qPCR with the number of colony-forming units (CFU) (10, 13), the number of *P. larvae* cells (14) or the number of *M. plutonius* cells (15). Such curves are based on various matrices (honey, larvae or crushed adult bees) experimentally contaminated with a known number of spores. The determined amplification cycle number, which is defined as the number of cycles required for the fluorescent signal to cross the threshold, was often referred to as the threshold cycle (Ct) or quantification cycle (Cq). In this review, it is referred to as Cq, in compliance with the guidelines for Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) (16).

Accurate pathogen quantification largely depends on qPCR sensitivity. Numerous studies underline the high sensitivity of the method (9, 10, 13, 14). In spite of small discrepancies, reported LODs are always very low, ranging from fewer than ten copies of the target gene (10, 14) to 37.5 vegetative bacterial cells per millilitre (10). Since the application of qPCR to the detection of *P. larvae* and *M. plutonius* is relatively recent, there is no consensus yet on the gene targeted for amplification. However, several researchers chose to target the 16S rRNA gene for both *P. larvae* and *M. plutonius* (9, 10, 15). Similarly, two different methods were used: SYBR® green I dye (9, 10, 14) or TaqMan® probes (13, 15). Although both methods give accurate results, the TaqMan® technology increases specificity since it includes a specific probe in addition to specific primers.

There are numerous applications of qPCR methodology. Two epidemiological studies performed in the United Kingdom and Switzerland – two countries where EFB is a notifiable disease – were designed to estimate the efficacy of sanitary measures on EFB control (13, 15). The accurate quantification of *M. plutonius* DNA in treated and untreated colonies showed that, although no treatment completely eradicated the bacteria in the apiary, the shook swarm method was more efficient than using antibiotics (15). Moreover, by comparing the bacterial loads of workers from brood nests with those of workers collected at the hive entrance, it was determined that the former were better indicators for disease surveillance (13). An interesting point in this work was the suggestion of setting a threshold for the emergence of clinical signs at 50,000 CFU/bee.

The issue of the best matrix for studying disease prevalence was also addressed for AFB (14). In this case, *P. larvae* DNA was quantified in experimentally infected drone and worker larvae. The authors concluded that the size of the larva is an essential parameter for a lethal threshold in AFB tolerance. The qPCR method was also used to quantify *P. larvae* spores and vegetative cells in honey (10).

Quantitative real-time polymerase chain reaction and nosemosis

Since 2009, about a dozen studies have described qPCR for detecting *Nosema* species. An accurate and fast method is needed to discriminate between *N. apis* and *N. ceranae* since their spores are too similar to be differentiated by optical microscopic analysis. Discrimination between spores of these two species can be performed by electron microscopy but this technique is not routinely used in honey-bee diagnosis. Moreover, colonies can be co-infected with both species (4, 17, 18, 19, 20, 21, 22), and some studies have suggested that the two species probably do not have the same virulence. Contradictory results have been published about virulence (see 8). To clarify this point, studies were

focused on determining the in-host competition of both species. One was performed using qPCR (22). Experimental co-infections followed by qPCR DNA quantification after 14 days showed no significant competitive advantage for either microsporidian.

It is essential to design primers specific to both species and judicious to develop methods to identify and quantify both microsporidia in a single reaction. In such cases, care must be taken to ensure a low LOD for the two species (6). Along these lines, several studies have described duplex qPCR reactions for the simultaneous detection and quantification of *N. apis* and *N. ceranae* (18, 19, 20, 21, 23, 24, 25). Only three studies used SYBR green (7, 18, 26); all the others used TaqMan® probes. The 16S rRNA region was used in all of these studies. Numerous studies also specified sensitivity. In two cases, the LOD reached ten copies of the target gene (19, 23). In all cases, standard curves were constructed to correlate a Cq value with a number of DNA plasmid copies (19, 20, 21, 22, 24, 25, 27) or with a DNA weight (18, 26). To underline the biological significance of the results, an effort was made to express them in spore equivalents per bee, calibrating the qPCR values to spore counts achieved by microscopy (23, 27). However, this estimation was biased as the authors evaluated the spore equivalents per bee on the hypothesis that the *N. ceranae* genome contains ten copies of the 16S rRNA gene. Even if qPCR performance has been correctly developed for specificity, sensitivity and reliability, the technique relies on an assumption (the number of gene copies), which undermines the results. No genomic analysis to date has confirmed this estimation. To overcome the difficulty of giving a spore equivalent per bee and of correlating spore counts with qPCR values (21, 24, 25), some studies gave their results in numbers of copies (20, 23, 24, 25) or simply in Cq (20).

Various studies have used qPCR analyses to identify and quantify *Nosema* species, including epidemiological studies or studies conducted at the colony, individual or organ level. The prevalence of nosemosis was determined in Virginia (United States [USA]) in 2009 (21), where the number of colonies infected with *N. ceranae* was found to be much higher than the number of colonies infected with *N. apis*. A higher load of *N. ceranae* than *N. apis* was found when the prevalence of *Nosema* species was compared in China, Japan, Chinese Taipei and the USA (19). It should be noted that Traver and Fell (21) emphasised the improved accuracy of qPCR methods at low infection levels compared to traditional spore counting, as they observed that, in half the colonies where no spores were found by microscopic analysis, *N. ceranae* DNA was detected by qPCR. Another epidemiological study compared the loads of *N. apis* and *N. ceranae* in 104 colonies in the USA (23). The precise quantification of both species over a year demonstrated that the load of *N. ceranae* carried by each honey bee was higher than that of *N. apis*. In a study designed to determine whether drones are naturally infected with *N. ceranae*, qPCR detected the presence of the microsporidian in drone pupae for the first time (24). Similar quantification for individual bees determined that there was no difference between *N. ceranae* loads on workers collected from honey supers (i.e. the part of the hive used to collect honey), those collected around the fringes of the brood nest or those within the brood nest itself (25). However, quantification revealed a seasonal effect for *N. ceranae*, the bees having been collected at various dates of the year (20, 24). A further degree of accuracy was reached with the quantification of *N. ceranae* alone (27), or concomitantly with *N. apis* (20), in honey-bee glands, the mid-gut or hind-gut. To date, no other molecular technique can detect both microsporidia simultaneously and specifically in bee organs.

Discussion

The development of new molecular techniques has become essential for honey-bee pathogen analysis. Numerous conventional PCR-based methods are used to detect bacterial and fungal honey-bee pathogens. However, the development of qPCR methods opens up new prospects. Many different samples can be handled concomitantly with qPCR, so it is feasible to develop a qPCR array to simultaneously identify and quantify numerous pathogens from the same sample. Such an array could be modelled on the qPCR array developed to analyse honey-bee diseases and immunity, which includes genes of the honey bee, *P. larvae*, *M. plutonius* and *N. apis* (28). The present review does not cover all the potential of qPCR because not all of its possibilities have been exploited yet. The qPCR technique can discriminate between two sequences differing by only one point mutation. This could be helpful when identifying closely related strains with distinct virulence properties. Another exciting prospect is the ability to detect and accurately quantify multiple pathogens through a single reaction. The qPCR technique has already been used to simultaneously detect *N. apis* and *N. ceranae*, but nowadays it is possible to identify and quantify up to five different pathogens within the same reaction, using probes labelled with five different fluorescent dyes. This would enable us to elucidate the multiple infections of honey-bee colonies by performing only a few reactions.

Genomics and transcriptomics could help to increase the potential of qPCR-based methods. Genomic analyses are needed to learn how many copies of qPCR target genes are found in the pathogen genome. The microsporidian genome, for example, usually contains numerous gene repetitions. It is thus crucial to know the number of gene copies of a given target gene, to enable us to correlate qPCR values with a spore number. Although numerous parameters may influence the outcome of a disease, such as strain virulence, natural host resistance and environmental

factors, this value would be helpful for determining a spore threshold for disease emergence, one piece of information still missing for foulbrood diseases and nosemosis. The determination of a spore threshold would enable improved decision-making about disease management.

Genomic tools are currently being developed for *N. ceranae* (29) and *P. larvae* (30, 31). Transcriptomic data would be useful for identifying genes that are specifically expressed at various stages of pathogen development, in mature or immature spores or in vegetative cells. Once these genes are identified, their relative expression in a sample could allow quantitative estimates of each developmental stage of the pathogen, and qPCR could then be used to determine the number of infectious forms of a pathogen in a sample.

In short, qPCR is a crucial tool that will inevitably be increasingly used in the future because of its unmatched performance. The development and more widespread use of qPCR methodology will lead to more reliable diagnoses and a better understanding of the pathogenic infection process. Disease control will be considerably facilitated. Like the conventional PCR-based methods that have become standard for identifying *P. larvae*, *M. plutonius*, *N. apis* and *N. ceranae*, qPCR methods must be validated, according to international standards. This is one of the objectives of the European Union Reference Laboratory for Honey Bee Health, which has already validated a qPCR method for the detection and absolute quantification of chronic bee paralysis virus (32). The ability to accurately quantify pathogen load in a colony is of major importance for determining a critical threshold in regard to clinical signs, especially for regulated diseases. Until now, detecting a pathogen but not being able to quantify it has necessarily entailed treating the disease. In the future, the measures to be taken will be targeted according to the pathogen level in a colony, i.e. whether this level can cause clinical signs and spread the disease to other colonies.

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Méthodes récentes de biologie moléculaire pour le diagnostic de la loque et de la nosémose

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Résumé

Le déclin des colonies d'abeilles mellifères est un problème de plus en plus préoccupant dans les pays occidentaux. Les causes de ce déclin sont nombreuses ; les infections par divers agents pathogènes en font partie. Plusieurs techniques de biologie moléculaire ont été mises au point pour détecter et identifier les agents pathogènes affectant les abeilles mellifères. La plus sensible, spécifique et fiable de ces méthodes est l'amplification en chaîne par polymérase quantitative en temps réel (PCRq). Les auteurs font le point sur les travaux publiés faisant état de l'utilisation de la PCRq pour détecter, identifier et quantifier quatre pathogènes majeurs des abeilles mellifères : les bactéries *Paenibacillus larvae* et *Melissococcus plutonius* (responsables de la loque américaine et de la loque européenne, respectivement) et les microsporidies *Nosema apis* et *N. ceranae* (responsables de la nosémose). Le recours à la PCRq pour détecter

les agents pathogènes des abeilles mellifères étant d'introduction récente, ces techniques vont certainement connaître une amélioration rapide ouvrant de nouvelles perspectives pour le diagnostic et la lutte contre les maladies. Ainsi, les techniques PCRq sont-elles appelées à devenir d'ici peu un outil puissant pour rechercher les causes de maladies infectieuses des abeilles mellifères et mieux comprendre les raisons du déclin des colonies d'abeilles.

Mots-clés
Abeilles – Abeilles mellifères – Amplification en chaîne par polymérase quantitative – Loque américaine – Loque européenne – *Melissococcus plutonius* – *Nosema apis* – *Nosema ceranae* – Nosémose – *Paenibacillus larvae.*

Métodos recientes de biología molecular para diagnosticar la loque y la nosemosis

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Resumen
La pérdida de colmenas de abejas melíferas constituye un problema de creciente gravedad en los países occidentales. En este fenómeno concurren muchas causas distintas, entre ellas las infecciones provocadas por diversos patógenos. Existen ahora técnicas de biología molecular que permiten detectar e identificar con fiabilidad a los patógenos de las abejas. El método más sensible, específico y fiable es la reacción en cadena de la polimerasa cuantitativa (PCRc) o PCR en tiempo real. Los autores repasan la bibliografía y describen varios estudios en los que se ha utilizado la PCRc para detectar, identificar y cuantificar cuatro de los principales patógenos de las abejas melíferas: las bacterias *Paenibacillus larvae* y *Melissococcus plutonius* (agentes causales de la loque americana y la loque europea, respectivamente) y los microsporidios *Nosema apis* y *N. ceranae* (agentes causales de la nosemosis). La aplicación de la PCRc a los patógenos de las abejas melíferas es muy reciente, y cabe prever que las técnicas vayan mejorando rápidamente y abran nuevas perspectivas para el diagnóstico y control de esas patologías. Así pues, las técnicas de PCRc podrían constituir en breve una potente herramienta para investigar infecciones y llegar a conocer y entender mejor el fenómeno de la pérdida de colmenas.

Palabras clave
Abejas — Abejas melíferas – Loque americana – Loque europea – *Melissococcus plutonius* – *Nosema apis* – *Nosema ceranae* – Nosemosis – *Paenibacillus larvae* – Reacción en cadena de la polimerasa cuantitativa.

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