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Minireview

Molecular Detection of Honey Bee Pathogenic Microbes: Recent Advances and Future Perspective

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Abstract Microbes, including bacteria and viruses, are the main threats to the health of honey bee colonies, and cause great losses to beekeepers. Rapid and accurate diagnosis is the key to controlling and eliminating honey bee diseases, and preventing them from spreading and causing an outbreak. This review summarizes recent advances in techniques to detect honey bee diseases, including traditional methods such as polymerase chain reaction, and next-generation sequencing methods, and how they are applied in the diagnosis and management of such diseases. It also discusses how these methods have revolutionized disease detection, and presents the future directions in the field of clinical diagnostics.

Keywords Honey bee, Detection, NGS, PCR, LAMP

INTRODUCTION

Honey bees play an important role not only in the production of bee products but also in sustainable agriculture, by pollinating a variety of crops and flowers, and preserving ecological balance and biodiversity in nature (Hung *et al*., 2018). However, these beneficial insects are susceptible to pathogens and the environment, which are important factors related to their heath. Honey bee pathogens include parasites, fungi, bacteria, and viruses (Moritz *et al*., 2010; Maggi *et al*., 2016). Among these, fungi, bacteria, and viruses are major pests in the beekeeping industry in most regions around the world, and cause significant economic losses since they mainly infect bee broods (Genersch, 2010a; Meixner, 2010; Potts *et al*., 2010; Smith *et al*., 2013; Chantawannakul *et al*., 2016).

Some fungi, such as *Ascosphaera apis*, *Aspergillus flavus*, *Nosema ceranae* and *N. apis* cause Chalkbrood, Stonebrood, and Nosemosis disease, respectively (Whitaker *et al*., 2011; Foley *et al*., 2014; Lee *et al*., 2016; Chen and Evans, 2021). In addition, two major bacterial diseases, American foulbrood (AFB) and European foulbrood (EFB), cause by *Paenibacillus larvae* and *Melissococcus plutonius*, respectively, and are highly contagious pathogens (Genersch, 2010b; Forsgren, 2010). The clinical symptoms of these diseases are confusing; the infections can spread on a wide scale, and are potentially lethal to infected colonies (Genersch *et al*., 2005; De Graaf *et al*., 2013; Forsgren *et al*., 2013; Gaggia *et al*., 2015; Khezri *et al*., 2018). Moreover, to date, 24 viruses have been identified in honey bees; in particular, 7 among them have high pathogenic capacity, including Acute bee paralysis virus (ABPV), Black queen cell virus (BQCV), Chronic bee paralysis virus (CBPV), Deformed wing virus (DWV), Israel acute paralysis virus of bees (IAPV), Kashmir bee virus (KBV), and Sacbrood virus (SBV) (Chen and Siede, 2007; Martin *et al*., 2012; Gisder and Genersch, 2015; McMenamin and Genersch, 2015; Ullah *et al*., 2021). Virus-infected bees exhibit a range of symptoms, from no overt signs of disease to rapid death and colony loss under certain conditions (Berényi *et al*., 2006; Chen and Siede, 2007; Gisder

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and Genersch, 2015; McMenamin and Genersch, 2015; Tantillo *et al*., 2015). Moreover, some honey bee viruses can be transmitted to other wild bees and vice versa through natural pollen sources (Fürst *et al*., 2014; Tehel *et al*., 2016; Radzevičiūtė *et al*., 2017; Tapia-González *et al*., 2019), increasing the risk of uncontrollable disease. Thus, fungi, bacteria, or viruses that cause rapid death to infected colonies or no overt signs are both critical challenges to beekeepers since effective treatments are not available. Hence, early detection of these pathogens is one of the main ways to control the development of the diseases. Furthermore, it is important to develop novel, rapid, and powerful detection methods to contain the spread of these pathogens before they cause a serious outbreak.

Previously, a wide range of methods was used to detect honey bee diseases, from observation and microscopy/electron microscopy to serological methods. However, these methods show some critical drawbacks such as confusing clinical signs, insensitivity, and lack of specificity. The introduction of nucleic acid/polymerase chain reaction (PCR)-based technologies has enabled improved sensitivity and specificity of detection of honey bee pathogens, especially fungi, bacteria, and viruses. Such technologies are dependent on accurate genomic information and the availability of suitable tools. The different approaches based on different biochemical principles, that can be used to detect one or more diseases in honey bees include: (i) multi-chain or PCR, multiplex PCR, DNA chip, real-time PCR, and next-generation sequencing (NGS).

This review gives a general idea of the available methods to detect honey bee infections.

1. Traditional detection

In the last few decades, diagnosis of honey bee diseases caused by microorganisms in the field was based on the visual inspection of brood-combs, odors, and changes in larvae color. However, these disease symptoms are easily confused with other diseases or brood abnormalities (De Graaf *et al*., 2013; Forsgren *et al*., 2013; Gaggia *et al*., 2015; Khezri *et al*., 2018). Moreover, viral diseases are typically not associated with clinical symptoms or may take asymptomatic forms, making diagnosis challenging (De Miranda *et al*., 2013; Amiri *et al*., 2018).

Culture methods and microscopy techniques have been used for the diagnosis of microbial pathogens in the laboratory. For a long time, the morphology of honey bee viruses have been examined by electron microscopy to determine their size and shape (Bailey, 1975; Bailey, 1983; Alippi, 1991). Some bacteria, including *P. larvae* and *M. plutonius*, have been successfully cultured to identify AFB and EFB, respectively (Allen and Ball, 1993; Schuch *et al*., 2001). Moreover, some fungal diseases have been classified by morphology (Jensen *et al*., 2013). These approaches have provided valuable information for identifying disease-causing agents in honey bees; however, they seem to be very insensitive, detecting less than 0.2% of microscopically counted bacterial cells (Alippi, 1991; Hornitzky and Clark, 1991; Hornitzky and Smith, 1998) and are limited by the slow growth rate of bacteria and the presence of undesirable microorganisms (Hornitzky and Smith, 1998; De Graaf *et al*., 2013; Forsgren *et al*., 2013). In addition, viruses are extremely small; hence, it is difficult to differentiate honey bee viruses using electron microscopy (Chen and Siede, 2007; De Miranda *et al*., 2013).

2. Immunological Techniques

1) Enzyme-linked Immunosorbent Assay (ELISA)

ELISA is a commonly used analytical biochemistry assay, first described in 1971 (Engvall and Perlmann, 1971). This method and its improved versions have been adapted for the detection of a number of honey bee diseases. EFB may have been the first disease to be detected and quantified using a polyclonal antibody; bacteria can be detected at 10^5 cells/mL, which is 100 times higher sensitivity than microscopy-based detection (Pinnock and Featherstone, 1984). Olsen reported that indirect ELISA with monoclonal antibodies was more sensitive and specific for the detection of AFB since it recognized *Bacillus larvae* (now *P. larvae*) but not six other *Bacillus* species (Olsen *et al*., 1990). Moreover, Anderson and colleagues performed a comparison among serological techniques to detect and identify some honey bee viruses, and confirmed that ELISA was the best method for sensitive and specific detection of BQCV, CBPV, KBV, SBV and honeybee

related viruses (Anderson *et al*., 1984). Recently, this technique was also applied to identify other honey bee viruses such as SBV and Thai SBV (Lee *et al*., 2010). ELISA is simple and fast, and allows the quantification of an antigen present in a certain sample. However, the procedures still have critical disadvantages such as expensive antibodies, sophisticated techniques, high possibility of false results, and antibody instability, which need to be improved for its application in field study (Aubert *et al*., 2008).

2) Agarose gel immunodiffusion (AGID)

The basis for AGID is concurrent migration of an antigen and antibody toward each other through agarose gel. When the antigen comes into contact with the complementary antibody, a precipitate is formed, and is trapped in the gel matrix, producing a visible line (Crowle *et al*., 1975). AGID is generally used to detect honey bee viruses (Anderson and Gibbs, 1989; Ribière *et al*., 2000; Todd *et al*., 2007) rather than bacteria or fungi. KBV and SBV were the first viruses to be detected by this method (Anderson and Gibbs, 1989), followed by most other honey bee viruses, including BQCV, SBPV (Slow bee paralysis virus), CBPV, ABPV, DWV, CSBV (Chinese sacbrood virus), and CWV (Cloudy wing virus) (Todd *et al*., 2007; Hu *et al*., 2016). The advantage of AGID is that it is a very simple, fast, and robust method that may be suitable for large-scale screening of viral infections. However, cross-contamination among viruses is an important problem that restricts the application of the AGID method. To avoid cross reactivity, monoclonal antibodies should be used, and this unfortunately raises the cost of the analysis. Moreover, the sensitivity of this technique is relatively low compared with those of molecular techniques (De Miranda *et al*., 2013).

Although ELISA and AGID have drawbacks, their immunological principle has successfully been applied to make some useful kits for quick screening of infections. Lateral flow devices (LFDs) are the best tools for field detection of several honey bee diseases. They were first reported to successfully detect *M. plutonius*, which is a causative agent of EFB, using antibodies, without cross reactivity with other bee pathogens (Tomkies *et al*., 2009; Mikušová *et al*., 2019a). To date, several types of LFDs have been commercialized to diagnose AFB or EFB, allowing more efficient detection and control of these diseases.

2. Molecular-based methods

1) PCR detection

PCR is a versatile method that allows for easy gene amplification (Mullis *et al*., 1986). It is also applied in genetic diagnostics, site-directed mutagenesis, and gene therapy, making it a popular and flexible technology. Many PCR tests and their variations, including reverse transcription (RT)-PCR, multiplex PCR, nested PCR, and real-time PCR, are widely used as quick and easy methods for the detection and identification of the causative agents of infectious diseases (Chen *et al*., 2004; Chantawannakul *et al*., 2006; Kajobe *et al*., 2010; Yoo *et al*., 2012a; Rivière *et al*., 2013).

KBV is potentially lethal to bees, and is associated with honey bee colony collapse disorder (Cox-Foster *et al*., 2007); it was the first virus of honey bees to be detected by an RT-PCR-based method with high specificity and sensitivity, as low as 1 pg of total RNA (Stoltz *et al*., 1995). Subsequently, a number of honey bee viruses were detected using RT-PCR, especially the 6 common viruses, KBV (Stoltz *et al*., 1995; Hung, 2000), ABPV, BQCV (Benjeddou *et al*., 2001), SBV (Grabensteiner *et al*., 2001), CBPV (Ribière *et al*., 2000), and DWV (Berényi *et al*., 2006; Ai *et al*., 2012; Ghorani *et al*., 2017). Pathogenic agents of bacterial diseases (AFB and EFB) in honey bees have been detected using PCR (Govan *et al*., 1998a; Govan *et al*., 1998b; Alippi *et al*., 2002; Alippi *et al*., 2004) and nested PCR assays (Djordjevic *et al*., 1998; Lauro *et al*., 2003). These methods allow rapid and specific detection of AFB and EFB in both agent cultures and directly infected bee hives.

2) Multiplex PCR detection

A PCR variant named multiplex PCR, which uses several sets of specific primers instead of one, allows more rapid detection of target genes of pathogens. In honey bee disease detection, multiplex PCR has been applied to simultaneously detect and differentiate two or three pathogens (Grabensteiner *et al*., 2007; Garrido-Bailón *et al*., 2013; Arai *et al*., 2014; Guimarães-Cestaro *et al*., 2016). The efficiency of multiplex

PCR or multiplex RT-PCR has been improved, and it can currently detect up to six viruses in honey bees simultaneously (Sguazza *et al*., 2013). This approach is very important in controlling diseases in honey bees, since in most cases, individual bees are commonly infected with multiple pathogens (Chen *et al*., 2004; de Miranda *et al*., 2010). However, multiplex PCR assays may fail to detect all the diseases in honey bees, owing to differences in gene expression of each disease, and are influenced by differences in primer annealing temperatures, concentrations of primers, and other parameters, all of which make it challenging to optimize such assays (Law *et al*., 2014).

3) Real-time PCR detection

PCR-based methods enable fast detection of honey bee diseases. However, conventional PCR yields results only after the final phase of the amplification, and does not allow accurate quantification, which is important to estimate pathogen load. Moreover, conventional PCR needs post-PCR handling such as gel electrophoresis, which increases labor time. Quantitative real-time PCR (qPCR) assays have been developed and validated for the detection of honey bee diseases. The first honey bees virus to be detected and quantified was DWV (Chen *et al*., 2005). Two common variants of qPCR have been developed based on amplicon detection methods: SYBR Green-based qPCR, and hydrolysis probe-based qPCR (TaqMan®). Although both methods give accurate results, the TaqMan® technology has higher specificity since it includes a specific probe in addition to specific primers (Mackay, 2004).

Owing to a number of advantages, including fast performance with high sensitivity, specificity, and reliability, real-time PCR has been used to detect various diseases in honey bees (Chantawannakul *et al*., 2006; Ward *et al*., 2007; Kukielka *et al*., 2008; Yoo *et al*., 2012a; Bradford *et al*., 2017; Schurr *et al*., 2017; Mataragka *et al*., 2020). Using qPCR, pathogens can be rapidly detected in a matter of minutes (Han *et al*., 2008; Truong *et al*., 2019). qPCR shows much higher sensitivity compared with conventional PCR and other methods, and it can detect as little as a few copies of target genes (Han *et al*., 2008; Kukielka and Sánchez-Vizcaíno, 2009; Blanchard *et al*., 2012; Yoo *et al*., 2012a; Bradford *et al*., 2017). Likewise, its variants, such as triplex qPCR, have been used to simultaneously detect honey bee diseases (Dainat *et al*., 2018); this method can also easily be combined with DNA chips (Kim *et al*., 2019b; Kim *et al*., 2020) or other methods (Kim *et al*., 2019a) for ultra-rapid and accurate detection.

Nevertheless, real-time PCR requires expensive instrumentation and special expertise to perform, and is hence not within reach of resource-constrained societies (Notomi *et al*., 2015).

4) Ultra-rapid real-time PCR detection

Ultra-rapid real-time PCR is a newly introduced technique to quickly and accurately detect pathogens (Cho *et al*. 2006; Han *et al*. 2008; Kim *et al*. 2010; Kang *et al*. 2011). This method is similar in principle to real-time PCR, but uses a glass-silicone-based micro-chip, which improves temperature homogeneity and heating conductivity, instead of PCR tubes. It successfully detects several honey bee diseases in a short time. *Paenibacillus larvae* was the first honey bee pathogen to be detected using this method; all the steps of detection were performed within 7 min and 54 s in a GenSpector® TMC-1000 system (Han *et al*., 2008). Following this, Yoo *et al*. reported that CBPV infection in honey bees could be detected in 16 min and 8 s (Yoo *et al*., 2010) and that 10^3 copies of SBV-bearing plasmid could be successfully identified within 17 min (Yoo *et al*., 2012a). Subsequently, other honey bee viruses such as IAPV, DWV, CBPV, ABPV, and SBPV were also detected using this method (Kim *et al*., 2019; Kim *et al*., 2019; Truong *et al*., 2019). Ultra-rapid real-time PCR can be used independently or in combination with DNA chips to enhance its specificity (Kim *et al*., 2020). This method is currently well established as a valuable tool for distinguishing honey bee diseases.

5) Reverse transcription loop-mediated isothermal amplification (RT-LAMP)

LAMP is a novel technique for nucleic acid amplification at isothermal conditions, yielding target DNA that can be amplified with inexpensive apparatus and unskilled personnel (Notomi *et al*., 2000; Sahoo *et al*., 2016). Moreover, this method requires at least 4 primers to recognize 6 different regions in the target DNA, which is expected to enhance specificity, efficiency,

and rapidity compared with that of other DNA-based detection methods (Notomi *et al*., 2000). LAMP produces typical DNA products that have cauliflower-like structures and can be visualized directly or using suitable dyes, by naked eye (Iwamoto *et al*., 2003; Mori and Notomi, 2009; Yoo *et al*., 2012b). Successful LAMP can be observed under normal light as white turbidity in the reaction tube, owing to extensive accumulation of magnesium pyrophosphate (Seetang-Nun *et al*., 2013), a by-product (Tomita *et al*., 2008; Jinlong *et al*., 2010), or as a change in the color of a pH indicator such as phenol red or cresol red, due to a dramatic decrease in pH in the reaction solution (Govan *et al*., 1998b; Yoo *et al*., 2012b; Tanner *et al*., 2015), or under UV light, in the presence of fluorescence reagents such as SYBR Green or Genfinder (Nguyen *et al*., 2011; Nguyen *et al*., 2012; Yoo *et al*., 2012b; Almasi *et al*., 2013). Moreover, LAMP and its variants have been developed for the detection of a number of diseases in honey bees in a very short time with high sensitivity, as low as 1 pg or even 10^3 copies of plasmid bearing the SBV target gene (Ma *et al*., 2011; Yoo *et al*., 2012b). In addition, LAMP can distinguish different viruses (Trzmiel and Hasiów-Jaroszewska, 2020; Dao *et al*., 2020). Therefore, this method is ideal for the development of a suitable tool for field-level diagnosis (Notomi *et al*., 2015; Wong *et al*., 2018). However, it should be considered that LAMP needs expertise in primer design, without which there is a risk of primer-primer interaction as well as cross contamination, and it is hence difficult to develop multiplex approaches (Higgins *et al*., 2018; Umesha and Manukumar, 2018; Wong *et al*., 2018; Trzmiel and Hasiów-Jaroszewska, 2020; Moehling *et al*., 2021).

6) NGS

Sanger sequencing technology has been used as the golden standard for nucleic acid sequencing, and has been widely applied to the diagnosis and molecular epidemiology of infectious diseases. It is expected to continue playing a vital role in disease prevention and control. This method generally targets either a single known pathogen or several individual pathogens. Investigating multiple infections in one or more hosts requires a variety of detection methods along with physical forces, and other modern equipment. Moreover, this method cannot be used to monitor and provide early warning of pathogens of other animal and viral diseases without appropriate sequencing.

NGS technologies have revolutionized research into infectious diseases. NGS has a wide range of applications, including research, clinical diagnosis, and epidemiology. Investigation of outbreaks and surveillance of infectious diseases have been greatly improved because the resolution of NGS is higher than that of other disease identification methods (Sikkema-Raddatz *et al*., 2013; Leopold *et al*., 2014; Hartman *et al*., 2019). The first application of NGS technology that demonstrated the potential of this approach for virus discovery was a metagenomic analysis of honey bees (*Apis mellifera*) adapted to shed light on the causes of colony collapse disorder (CCD), which strongly correlated with IAPV (Cox-Foster *et al*., 2007). More recently, NGS has been applied to investigate AFB epidemiology in honey bees, to determine when and from where the pathogen was introduced (Ågren *et al*., 2017). Excitingly, NGS has been used to identify a number of infections as well as reveal new viruses in honey bees (Ågren *et al*., 2017; Haddad *et al*., 2018; Kraberger *et al*., 2019).

However, NGS has several limitations, including a requirement for sophisticated operating system and bioinformatics software expertise in data analysis, and large data storage capabilities. In addition, expensive cost for NGS apparatus and library preparation are challenges for many developing countries. Therefore, this technology is still at the research level, and more time is needed to reduce its cost and simplify it for application in routine clinical practice (Yohe and Thyagarajan 2017; Gu, 2019).

DISCUSSION

Bacterial and viral diseases are major threats to honey bees, since they are generally deadly, highly contagious, and often lack obvious symptoms. Many avenues have been developed to control honey bee diseases, including detection methods utilizing a number of different techniques. Culture-based methods and microscopy are time-consuming and less sensitive, and immunology-based methods require specific antibod-

ies, which are expensive. In contrast, LFDs are good tools for field detection of some honey bee diseases. However, LFDs are not available for most honey bee viruses, although they seem to be the best strategy for beekeepers. Molecular-based methods have shown very good performance and have accurately identified diseases in honey bees; however, they have some limitations, such as easy contamination during sample preparation, and nonspecific binding.

In addition to the techniques mentioned above, other technologies have been developed in the field of honey bee disease detection, including microarrays (Glover *et al.*, 2011), in situ hybridization (Yue *et al.*, 2008; Park *et al.*, 2016), mass spectrometry (Tokarz *et al.*, 2011; Aliferis *et al.*, 2012), biosensors (Mikušová *et al.*, 2019b), and barcode sequencing (Erban *et al.*, 2017). Despite some limitations, NGS technologies are powerful and promising for clinical diagnosis of honey bee diseases in the future, since they generate genomic and transcriptomic data, which would be useful for other methods to identify target genes and determine the expression levels of virulent genes of pathogens. Real-time PCR is an important tool that will certainly be increasingly used in the future because of its unprecedented performance. More extensive development and use of newly developed qPCR methods will lead to more reliable diagnostics and a better understanding of the infection process. This will significantly facilitate the control of infectious diseases in honey bees and other commercially important animals. Along with conventional PCR-based methods, qPCR has become the standard for identifying bacteria and viruses that cause damage to bee health and populations. The ability to accurately quantify the number of pathogens in a hive is of great importance to determining the threshold of importance associated with clinical signs, especially for the role of disease. To date, diseases have been treated by detecting pathogens without quantifying them. In the future, measures taken will be targeted at the pathogen level in a hive; this level may cause clinical signs and spread the disease to other hives.

In summary, many techniques that can be applied to honey bee disease detection are under development. To achieve a highly accurate assay, a combination of methods may be necessary, in order to exploit the advantages of each method and improve the efficacy of pathogen detection (Borum *et al.*, 2015; Sopko *et al.*, 2020).

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DISCLOSURE STATEMENT

The authors report no potential conflict of interest.

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