

## PRELIMINARY STUDIES INTO NOVEL DETECTION METHODS FOR HONEYBEE PATHOGENS

Ruth WAITE<sup>1</sup>, Helen THOMPSON<sup>1</sup>, M. BROWN<sup>1</sup>, M. WATKINS<sup>2</sup>, M. BEW<sup>1</sup>

<sup>1</sup>National Bee Unit, Central Science Laboratory, Sand Hutton, York YO41 1LZ, ENGLAND

<sup>2</sup>Vita (Europe) Ltd, 21 Wote Street, Basingstoke, RG21 7NE, ENGLAND

### Abstract

The National Bee Unit, part of the Central Science Laboratory (an executive agency of the Department for Environment, Food and Rural Affairs, Defra) has been developing novel techniques for detection of bee pathogens. Two different methods are under examination, an antibody-based test for identification of foulbrood diseases for use in the field, and a nucleotide-based laboratory test for viruses and differentiation of bee species.

The foulbrood field test kits are to detect the pathogenic micro-organisms *Melissococcus plutonius* and *Paenibacillus larvae* subsp. *larvae*. Monoclonal antibodies (IgG) were developed at CSL, using freshly isolated bacterial cultures obtained from infected material. The kits (termed lateral flow devices) are based on existing technology owned by CSL. Antibodies underwent vigorous screening for specificity to the relevant bacterium, testing for cross-reactivity with other bacteria commonly found in honeybee colonies, including *Paenibacillus alvei* and *Brevibacillus laterosporus*. Suitability for use in the lateral flow devices was also tested. The kits for AFB were validated in the laboratory in 2002, ready for field testing in 2003, and those for EFB are under development, with anticipated laboratory testing in 2003.

The technique for detection of viruses and different bee species is based on a real-time PCR method called Taqman®. Genetic sequences for many bee-related viruses are available and primers to certain viruses (including Kashmir Bee Virus and Acute Paralysis Virus) have been designed to detect them from honeybee samples. Although in early stages, indications so far are very promising. The methodology has a high sample through-put, with potentially 1500 tests achievable each week, detecting four different viruses simultaneously. This technology thus has great potential for surveillance projects both in the UK and overseas. A DNA probe capable of identifying Africanised honeybee DNA has also been developed, in order to survey for Africanised bees.

### Introduction

Despite major advances in the field of disease diagnostics, the methods used for routine bee disease diagnoses are based on traditional methodologies such as basic microscopy or serological testing. The National Bee Unit (NBU), part of the Central Science Laboratory (CSL) (an executive agency of the Department for Environment, Food and Rural Affairs, Defra) has been investigating novel techniques for rapid detection of bee pathogens. Two different methods are under examination, an antibody-based field test for identification of foulbrood diseases, and a nucleotide-based laboratory test for viruses and differentiation of bee species. The intention of this paper is to introduce these newer methods for bee disease diagnosis and explain the progress made to date.

Honeybees are afflicted by relatively few diseases, which usually affect either adults or larvae specifically. Two such diseases are American foulbrood and European foulbrood, both of which affect larvae and are caused by bacteria. The occurrence of these diseases has considerable economic impact on the bee industry, as bees are valuable with respect to pollination and production of honey and beeswax (CARRECK and WILLIAMS, 1998). Both diseases have been described and reviewed in detail elsewhere (SHIMANUKI, 1983, 1990; RATNIEKS, 1992; HANSEN & BRØDSGAARD 1999). American foulbrood (AFB) is caused by *Paenibacillus larvae* subsp. *larvae* (HORNITZKY, 1998), previously known as *Bacillus larvae* (HEYNDRICKX *et al*, 1996), an aerobic spore-forming bacterium. European foulbrood (EFB) is caused by the fastidious microaerophilic bacterium *Melissococcus plutonius*, formerly *Melissococcus pluton* (BAILEY and COLLINS, 1982; BAILEY, 1983; TRÜPER and DE' CLARI, 1998). However, there are usually other bacteria found in EFB-affected larvae, such as *Paenibacillus alvei* and *Brevibacillus laterosporus*, both thought to be secondary saprophytic organisms (ALIPPI, 1991).

Both brood diseases occur throughout the world although AFB is generally of greater interest. EFB is of particular concern in the UK, but has relatively little impact elsewhere (SHIMANUKI, 1990; THOMPSON and BROWN, 2001). Under UK Bee Health legislation (The Bees Diseases Control Order, SI 1982 No. 107, 1982), both diseases are notifiable and must be reported to the relevant authorities if found. In England and Wales, the NBU co-ordinates the bee health programmes for both Defra and the National Assembly for Wales Agriculture Department (NAWAD). The NBU has a team of Appointed Bee Inspectors (ABIs) that inspects colonies throughout England and Wales. If a colony is suspected of having either foulbrood disease, a symptomatic larval sample is taken and sent to the NBU diagnostic laboratory, where the sample is examined for pathogenic bacteria for disease confirmation.

A test kit capable of confirming disease in the field without sending samples to the laboratory has obvious benefits. It would allow ABIs to confirm their diagnosis immediately during an inspection, resulting in more efficient and effective disease control. It would also enable laboratory diagnostic staff to undertake more research work and expand into other areas of bee health, such as surveillance for exotic pathogens. CSL has over 600 scientists with expertise in diverse disciplines, such as molecular biology, analytical chemistry and insect pathology. Within the organization there is a team devoted to rapid disease diagnosis, including development of field test kits, primarily for plant diseases. These kits, termed lateral flow devices

(LFDs), were developed for immediate field diagnosis of plant viruses, such as the potato viruses X and Y (DANKS and BARKER, 2000). Our investigation aimed to adapt this technology for foulbrood diagnosis kits for use by both bee inspectors and beekeepers.

Further problems in honeybees are thought to be associated with the presence of viruses. There are various techniques available, including gel immunodiffusion or ELISA-based assays using polyclonal antibodies (ANDERSON, 1984; TODD and BALL, 2003). Methods currently available are suitable for heavily infected samples or small numbers of samples, but cannot easily be applied for use in large-scale surveys, or to detect low levels of virus, such as might be found in inapparent infections. In addition, sensitive and specific serological methods for detection of honeybee viruses are difficult to develop as many preparations of honeybee viruses are mixtures; most colonies contain one or more viruses (BAILEY et al., 1981; STOLTZ et al., 1995; EVANS and HUNG, 2000). It is therefore difficult to produce truly specific antisera to each bee virus (ANDERSON, 1984).

Many bee-related viruses have been sequenced in recent years, with sequences deposited in publicly accessible databases such as GenBank and EMBL (GHOSH et al., 1999; GOVAN et al., 2000) and the nomenclature tentatively established (EVANS and HUNG, 2000; MAYO, 2002). However, there have been few surveys of their incidence (ALLEN and BALL, 1996). The increased availability of viral sequences has enabled the NBU to pursue a novel technique for virus identification called TaqMan<sup>®</sup>, based on real time-PCR (RT-PCR).

## **Materials and Methods**

### ***Lateral flow devices***

#### *Initial investigations*

The field testing kits, termed lateral flow devices, are designed to detect the pathogenic bacterium associated with AFB (*P. larvae* subsp. *larvae*). Several monoclonal antibodies were developed in-house at CSL and screened for specificity to *P. larvae* subsp. *larvae*. After this initial screening, an antibody was chosen as most suitable due to its activity, fitness for use in the LFD and lack of cross-reactivity against other hive-related bacteria, including *M. plutonius*, *B. laterosporus*, *P. larvae* subsp. *pulvifaciens* and *P. alvei*. Once screened and found to be specific, it was introduced into the LFD and further work was undertaken.

#### *Laboratory validation*

Validation in the laboratory involved blind-testing many different samples (which are routinely sent to the diagnostic laboratory as part of the NBU inspection service) to see if the antibody was completely specific for *P. larvae* subsp. *larvae*. Although most samples tested were infected with one of the foulbrood diseases, others were included, such as chalkbrood mummies and healthy larvae.

### ***TaqMan<sup>®</sup> for identification of viruses***

#### *Initial investigations*

In order to carry out detailed comparisons of *inter* and *intra* viral sequence variability the coat protein genes for *Kashmir bee virus* (KBV), *Acute bee paralysis virus* (ABPV), *Sacbrood virus* (SBV) and *Black queen cell virus* (BQCV) were downloaded from the EMBL sequence database. Multiple sequence alignments were carried out using the CLUSTAL V algorithm in the package Megalign (DNA star). Phylogenetic analysis was then carried out by calculating genetic distances between pairs of sequences using the Jukes and Cantor algorithm, and clustering was done from these matrices by neighbour joining in TREECON (VAN DE PEER and DE WACHTER, 1994). The statistical significance of the branching was estimated by performing 100 replications of bootstrap resampling from the original data. For *Cloudy wing virus* (CWV), sequence is only available in the replicase gene not in the coat protein gene. The sequence for the RNA dependent RNA polymerase (replicase) gene was compared to the replicase gene for *Kashmir bee virus* (KBV); other replicase sequences are unavailable.

#### *TaqMan<sup>®</sup> probe and primer design*

The design of primers and probes for the TaqMan<sup>®</sup> assays were carried out using the Primer Express<sup>™</sup> software (PE-Biosystems), as described in Mumford *et al* (2000). Forward and reverse primers and a FAM-labelled probe were designed for KBV, CWV, SBV and BQCV. The regions of sequence selected for assay design were those where there was a large degree of variation between viral species, but a large degree of conservation within species. An internal positive control assay (IPC) was designed to the

18S ribosomal gene of *Apis mellifera*. The probe for this assay was labelled with VIC rather than FAM and thus could be used in multiplex with any of the virus assays designed. This control allowed the monitoring of RNA extraction efficiency from samples and guarded against false negative results (i.e. where no virus is detected due to failure to extract RNA from the bee samples).

### *TaqMan*<sup>®</sup> assays

*TaqMan*<sup>®</sup> reactions were set up in 96-well reaction plates using PCR core reagent kits (PE-Biosystems), following the protocols supplied, but with the addition of 25 units of M-MLV (Promega) per reaction. For each reaction, 1 µl of RNA extract was added, giving a final volume of 25 µl. Plates were then cycled at generic system conditions (48°C/30 min, 95°C/10 min and 40 cycles of 60°C/1 min, 95°C/15 sec) within the 7700 or 7900 Sequence Detection System (PE-Biosystems), using real time data collection.

## Results

### *Lateral flow devices*

#### *Initial investigations*

Several antibodies were developed with activity against *P. larvae* subsp. *larvae*. The most promising were screened and one chosen for further study. It was completely specific for *P. larvae* subsp. *larvae*, showing no reactivity against any of the following bacteria: *M. plutonius*, *B. laterosporus*, *P. larvae* subsp. *pulvificiens*, *P. alvei*, an unidentified anaerobic bacterium isolated from an EFB-infected sample, *Escherichia coli* or *Ralstonia solanacearum* (the cause of potato brown rot). This antibody was introduced into an LFD and found to be suitable.

#### *Laboratory validation*

Table I gives a summary of the results of the laboratory validation with the AFB LFD.

Table I

Summary of AFB LFD laboratory validation tests

Sample type	Total number tested	Reacted with LFD
AFB positive sample	77	71
EFB positive sample	87	1
Other eg chalkbrood mummy	31	0

The results indicate that the LFD test was highly specific for AFB-infected larvae. There was just one false positive reaction, which was on a previously frozen larva infected with EFB; this showed a weak positive reaction. As there was just this one false positive, it was thought that this was a singular occurrence unlikely to happen again. The samples that were diagnosed originally as AFB-positive but were missed by the LFD kit were very dilute; these results were not surprising as kits are designed to detect the high number of spores present in a symptomatic larva. There were no reactions with other larvae tested, such those containing *P. alvei* or *B. laterosporus* or those that were apparently healthy from the same combs as other larvae affected with AFB or EFB.

### *TaqMan*<sup>®</sup> for virus identification

#### *Sequence analysis*

By comparing the sequences of the viruses of interest, regions were selected that would give good separation of the virus species. Of particular note are *Kashmir bee virus* and *Acute bee paralysis virus*: these two species are closely related although following analysis was shown that the coat protein could be used to discriminate them. We were unable to obtain a purified sample of ABPV to show lack of cross-reactivity. The only sequence available for *Cloudy wing virus* is the replicase gene. Following pair-wise sequence comparisons it was shown that the replicase gene of *Cloudy wing virus* and *Kashmir bee virus* were identical and the assay designed based on this sequence was expected to also detect *Kashmir bee virus*.

TaqMan® assays

The TaqMan® assays designed were tested on a range of purified virus preparations acquired from CSIRO, Australia. Results are shown in figure 1.

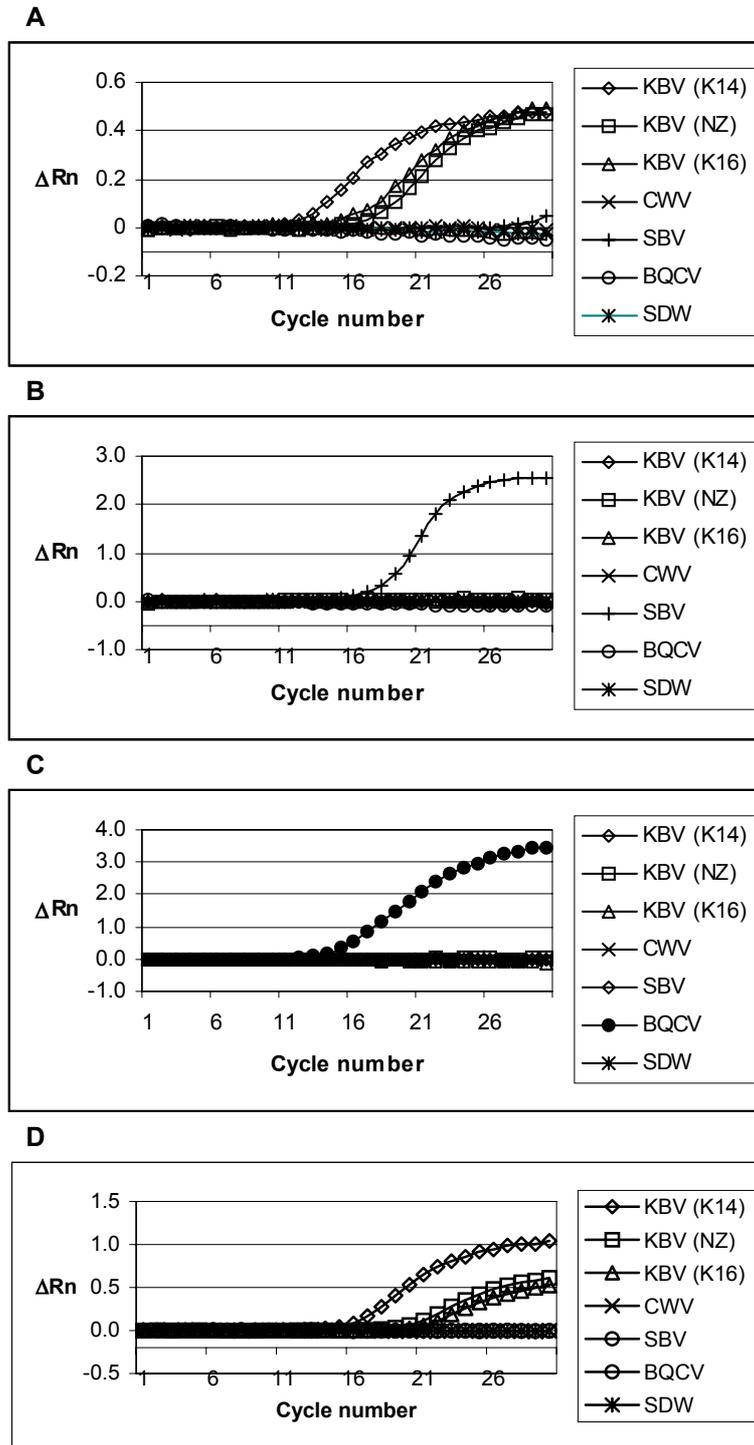


Figure 1 - Illustrating the detection of bee viruses using real-time PCR.  
 A: Detection of KBV with the KBV assay, B: SBV with the SBV assay,  
 C: BQCV with the BQCV assay and D: detection of KBV with the CWV assay.  
 SDW is the negative control spiked with water.

In each case, it was shown that the assay gave the expected result. The assays for *Sacbrood virus* and *Black queen cell virus* are completely specific, whilst the assays for *Kashmir bee virus* and *Cloudy wing virus* both detected all isolates of *Kashmir bee virus*. Neither assay however detected any RNA in the

*Cloudy wing virus* preparation; further investigation is needed before the reasons for this can be confirmed, although it is suspected that the RNA in the preparation has degraded. The assay for *Kashmir bee virus* was apparently specific for the isolates of KBV.

## Discussion

The methods detailed in this paper are novel in the field of honeybee disease diagnosis, although they have been used successfully for plant diseases for several years (MUMFORD et al., 2000; DANKS and BARKER, 2000). However, they have been adapted successfully for use in honeybees.

The field test kits for American foulbrood are a completely novel concept for bee disease diagnosis. In countries where bee diseases are diagnosed, this is routinely carried out in a laboratory (ALIPPI, 1991; OIE, 2000). The ability to undertake this in the field is of great interest especially in countries where it is impossible to have a comprehensive inspection service, such as Australia (GOODWIN, pers. comm.). Kits for the detection of European foulbrood are also under development and are currently at the laboratory validation stage. It is anticipated that the field validation stage will be initiated before the end of the 2003 season in the UK. It is this disease that is of most interest in the UK (THOMPSON and BROWN, 1999).

Detection of viruses using the TaqMan<sup>®</sup> technique has enormous potential for future work both in the UK and abroad. Any surveys with respect to the occurrence of bee viruses in colonies to date have been dependent upon access to antisera. Thus, comparison of data has been dependent on the specificity of the antisera generated in different laboratories. This is of particular importance as there are often multiple viral infections in bees and colonies (EVANS, 2001). Quantitative ELISAs similarly also require access to suitable antibodies and purified virus preparations (TODD and BALL, 2003). In most cases, surveys have not been able to detect inapparent infections and are used primarily in a diagnostic function (HORNITZKY, 1987; ALLEN and BALL, 1996). Due to the methodologies involved, they are also limited in their sample sizes (TODD and BALL, 2003) and may not always provide conclusive results (RIBIERE et al., 2000). However, as this study has shown, the use of TaqMan<sup>®</sup> technology overcomes issues of reagent availability, enables the application of highly sensitive and specific RT-PCR to be applied quantitatively (over several orders of magnitude) to large numbers of samples, and the use of an internal 18S ribosomal gene control assesses efficiency of extraction ensuring false negatives do not occur.

It is evident that these new technologies can be applied successfully to the detection of honeybee diseases. Further work is anticipated in both areas reported in this paper. This could lead to a new era in the understanding of some of the unknowns in the often mysterious world of these infections, and perhaps to better ways of disease management.

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