The Foster method: Rapid and non-invasive detection of clinically significant American Foulbrood disease levels using eDNA sampling and a dual-target qPCR assay, with its potential for other hive pathogens.

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Abstract

Clinical signs of American Foulbrood can be difficult to diagnose and thus disease is missed and leads to further spread. Diagnosis is centred on the beekeeper's skill in recognising clinical symptoms – a highly subjective and time-consuming activity. Previous testing methods have relied on sampling that necessitates dismantling the hive and/or requires multiple visits to retrieve passive samples. The Foster method is a novel environmental DNA sampling method using entrance swabs together with a dual-target qPCR for AFB. The quantification data generated can be used to detect hives with clinically significant infections, even before visual symptoms are apparent. Such a method will be applicable to other bee pathogens and incursion pests.

Keywords: honey bee, American foulbrood, entrance, qPCR, diagnosis, swab, quantification, non-invasive.

Importance

Discovery of the disease American foulbrood typically means the destruction of the bees and hive by burning. This discovery is typically by visual examination of capped brood by the beekeeper - a subjective skill that means the disease is being missed or not recognised. It is a time-consuming and exacting process to inspect hives for AFB. Here we present a rapid sampling method that does not require opening/ dismantling the hive, in conjunction with a dual target quantitative PCR assay for the bacteria responsible, *Paenibacillus larvae*. Using the resulting quantitative data, hives presenting clinical symptoms or likely to become clinical visually can be determined and the hives dealt with appropriately before further spread occurs.

Introduction

American Foulbrood (AFB) is one of the most destructive diseases in honeybees (*Apis mellifera*) and is caused by spores of the Gram positive bacteria *Paenibacillus larvae* infecting bees during their larval stage. These spores are extremely hardy; being resistant to heat, caustic, and other chemical treatments with the ability to remain infectious for over 30 years (Genersch, 2010). Only a few spores are required to cause an infection in a new larva and yet a single infected larvae can generate many millions of spores - leading to rapid spread and decline in a bee colony. Once the colony has weakened or died then it may be 'robbed' by bees from neighbouring hives where contaminated honey is brought back to a neighbouring hive, which leads to further infections. Infection is also spread unknowingly by the beekeeper (Fries and Camazine, 2001), through the introduction of hive frames carrying an undetected infection into a new colony of bees or the feeding of contaminated honey to colonies.

In New Zealand, hive numbers have more than doubled since 2013 (www.afb.org.nz). Similarly the number of beekeepers has also increased – primarily due to hobby and conservation efforts (several hives per beekeeper) but also in the commercial sector (hundreds or thousands of hives per operation). Much of the growth in hive and commercial beekeeping has been due to the increased demand for high-value New Zealand manuka honey. The increase in hive numbers (and densities) combined with newer beekeepers, who are less likely to be skilled in identifying the clinical signs of AFB, has led to an increase in the percentage of hives reported by beekeepers as infected from 0.21% in 2015 to 0.32% in 2017 (www.afb.org.nz). Since this time, the reported percentage has remained static, while hive numbers have continued to increase. (www.afb.org.nz).

The diagnosis of AFB is based primarily on visual symptoms – typically unusual, capped brood cells on a beehive frame that warrant investigation through a ‘roping’ field test. In a hive with three to seven frames of brood or more, this means typically thousands of brood cells to examine visually per hive. Other diseases can affect brood cells meaning that examining suspicious cells for clinical AFB signs is laborious and prone to disease being missed. Clinical signs can vary based on the AFB genotype present, with ERIC II strains exhibiting reduced clinical symptoms (Genersch, 2010). In addition, hives must be dismantled, and the colony disrupted in order to inspect for AFB.

Confirmation for AFB in beehive materials has been traditionally performed by culture, using selective media, and induced germination of spores. However this is a variable process (Forsgren, 2008) and may take up to a week for results. PCR and qPCR (quantitative PCR) have been demonstrated as effective tools for the detection of AFB, with qPCR being the preferred diagnostic tool in recent years due its faster time to results and lower chance for amplicon contamination. However most applications have not used the quantitative data generated; rather using the tool for rapid confirmation of culture isolates (Kňazovická et al, 2011). The quantification aspect is important as low levels of AFB are often not clinically relevant (Bassi et al., 2018 , Pernal and Melathopoulos, 2006) and such hives may never develop clinical symptoms. Much of this low level may be due to the hygienic behaviour of bees to reduce overt infections (Spivak and Reuter, 2001).

The vast majority of molecular publications relating to AFB to date have relied on either conventional PCR or SYBR Green-based qPCR and the sole use of the 16S gene due to its high sensitivity as a multicopy target (Dobbelaere *et al.*, 2001; Piccini *et al.*, 2002;). However this sensitivity can be at the expense of specificity, as

evidenced by the initial design (Martinez, 2010) and subsequent re-evaluation/re-design of qPCR primers to improve specificity (Rossi *et al.*, 2018). Sensitivity for bacterial targets can also be compromised by deletions of target sequences or sequence polymorphisms which affect primer or probe binding (Dahlberg *et al.*, 2018; Johansen *et al.*, 2019; Xiu *et al.*, 2021) and many microbiological qPCR tests now utilise two independent targets to enhance both sensitivity and specificity, such as many of the commercial qPCR tests for chlamydia. Hydrolysis probe ("TaqMan") qPCR assays permit quantification of each target by selected fluorescent wavelengths while also allowing the use of an internal control in an additional detection channel for confirmation of suitable quality DNA present in the reaction. These internal controls may improve the sensitivity of previous studies whereby the presence of inhibitors possibly prevented the amplification of AFB targets. (Forsgren and Laugen, 2013)

We sought to use a multiplex qPCR assay for AFB and evaluate a new and rapid environmental DNA (eDNA) sampling method for the detection of hives having - or at risk of developing - clinical AFB infections. Previously AFB has been detected in debris from a hive baseboard (Bassi et al., 2018) but this study required installing collection sheets, necessitating dismantling the hive twice as well as repeat visits. The ability to detect relevant levels of AFB from a single sample taken from the entrance of a hive would permit far more rapid sampling of hives and allow infected hives to be dealt with more quickly (Lyall et al., 2019). In this study, we evaluated and compared the AFB levels among bees, the entire baseboard, and the entrance region of the baseboard.

Methods

Quarantining hives in an apiary upon discovery of a clinical AFB hive is a common international practice, in order to minimise spread of the disease. From five quarantine apiaries around New Zealand (East Coast, Wellington, Wairarapa and South Canterbury regions) multiple hives were sampled two-monthly from late 2018 to early 2020. In addition, samples from visually clinical hives and neighbouring hives were collected by local apiary inspectors for testing during this time. Samples included nurse bees, swabs from the hive entrance and swabs from over the entire baseboard. Subsequent samples comprised of comparisons between bees and solely the hive entrance. Bees were collected from the brood frames using 50 mL sterile containers and frozen to euthanise the bees. Sterile foam-tipped swabs (Puritan) were moistened in sterile water before swabbing either the whole baseboard or just through and across the hive entrance for three to five seconds. Instructions were provided to participating beekeepers to rotate the swabs during collection to ensure all swab surfaces could collect hive material. Swab heads were then snapped off into a 2 mL microcentrifuge tube, capped and sent to the laboratory with the bee samples, at ambient temperature by overnight courier. Once in the laboratory, bee samples were frozen at -20°C and swabs were stored at ambient temperature until DNA extraction was performed.

Genomic DNA extraction from bees and associated bacteria was performed from each sample containing 10 bees. DNA was extracted using the Bee Pathogen DNA/RNA Extraction Kit (dnature diagnostics & research Ltd, Gisborne, New Zealand) in conjunction with beadbeating. 10 bees were used for the practical purpose of ease of sample handling and throughput. In short, 3 mL DXL lysis buffer supplied in the kit was added to 10 bees in a 5 mL tube, containing a mixture of 0.5

mm (~2.4 g) and 2.3 mm zirconia silica (~1 g) beadbeating beads (BioSpec Products, Bartlesville, USA). The bees were homogenised in a Mini Beadbeater 16 instrument (BioSpec Products, Bartlesville, USA) for 3 minutes. Tubes were then incubated at 65°C for 10 minutes with manual inversion every 3 minutes. 1 mL of the homogenate was transferred to a 2 mL microcentrifuge tube and centrifuged at 15,000 x g for 5 minutes. 500 µL of supernatant was transferred to a new microcentrifuge tube containing 450 µL AD buffer and mixed well. The solution was then applied to a nucleic acid binding column from the kit (in two passes) and centrifuged through before successive washing steps and elution of nucleic acids from the column in 50 µL elution buffer.

A similar process was used for swabs: they were placed in a screwcap 2 mL tubes with 0.5mm zirconia silica beads (~0.8g) and 2.3 mm zirconia silica beads (0.3g). 800 µL DXL buffer was added to each tube and the swab material homogenised in the Mini Beadbeater 16 for 3 minutes. The tubes were then incubated at 65°C with shaking for 10 minutes, before centrifuging and processing 500 µL of the resulting supernatant as for the bees. For pooled applications, swabs were added to 500 µL DXL buffer in a 2 mL microcentrifuge tube and vortexed for 10 seconds to dislodge cells and bacteria off the swab. 60 µL of each eluate (up to twelve swabs) was added to a 2 mL beadbeating tube as for single swabs and processed as for single swabs from the beadbeating step.

Assessment of DNA quality and presence of potential inhibitors was performed by measurement (spectroscopy) on a DS-11 FX (DeNovix, Wilmington, USA) and OD260/280 measurements recorded. Dilutions of extracted DNA were also tested by qPCR to measure the expected Cq increases with increasing dilution and thus monitor any qPCR inhibition.

The AFBduo qPCR test (dnature diagnostics & research Ltd, Gisborne, NZ) uses two targets – a single copy gene for quantification purposes (ftsZ, a prokaryotic homologue to tubulin) and the multicopy 16S gene. The ftsZ probe is detected in the FAM channel and the 16S target in the HEX channel. The test also includes a simultaneous internal control (ROX channel) for an animal mitochondrial marker, to confirm validity of results (in the cases where AFB was not detected) by way of successful internal control amplification.

Each AFBduo qPCR reaction was performed in a 10 µL volume and consisted of 5.5 µL PCR grade water, 0.5 µL of the 20X Oligo Mix and 2 µL of the 5X Mastermix (all reagents supplied in the AFBduo real-time PCR kit, dnature). 2 µL genomic DNA, positive control DNA or sterile water (for no template controls) was added per reaction. The amplifications were performed on the Mic qPCR Cycler (BioMolecular Systems, Australia) or the Eco qPCR instrument (Illumina, San Diego, USA) and the cycling conditions comprised: denaturation at 95°C for 2 minutes followed by 40 cycles of 95°C for 5 seconds and 60°C for 15 seconds. Data was acquired on the Green (ftsZ), Yellow (16S) and Orange (Internal control) channels of the Mic cycler or FAM, HEX and ROX channels on the Eco instrument. Cq's (Cycle quantities) were automatically generated by the Mic software, using dynamic analysis.

A synthetic DNA standard (gBlock, IDT, Singapore) was utilised that incorporated both amplicon sequences. The synthesised standard was resuspended in TE buffer, pH8, quantified on a DS-11 FX spectrophotometer (DeNovix, Wilmington, USA) and its copy number calculated using the equation:

$$\text{Copies} = \frac{\text{ng} * \text{Avogadro's constant copies/mole}}{\text{length of standard} \times 660 \text{ g/mole} \times 1\text{x}10^{9} \text{ ng/g}}$$

Quantification standards were generated from 10^6 copies per µL down to 2 copies per µL. For all dilutions, the diluent was a pooled DNA sample extracted from AFB-free bees. Dilutions of extracted DNA from AFB-positive samples were diluted in AFB-free bee DNA. These dilutions were used to compare the sensitivity among existing World Organisation for Animal Health (OIE) conventional PCR protocols (Dobelaere, 1999; Govan, 2003) and the AFBduo qPCR assay. Conventional PCRs used the same 2 µL each DNA dilution in a 20 µL PCR using HOT FIREPol polymerase, ready to load (Solis BioDyne, Tartu, Estonia) and 0.3 µM each primer. Following the recommended thermalcycling conditions (Dobelaere, 1999; Govan, 2003), the conventional PCR reactions were analysed by electrophoresis on a 1% agarose gel.

The standards and their resulting Cq's were also used to convert the Cq's resulting from bee and swab samples to spore levels using the amplification efficiency of the single copy ftsZ gene, the y-intercept, and the formula:

Spores per swab / 10 bees = $10^{(Cq\ ftzZ-39/-3.48)} \times 25$

Results

Testing of the AFBduo assay on synthetic DNA standards showed high analytical sensitivity, with both target reactions in the assay able to reliably detect down to 4 copies per 10 µL reaction. The reaction targets amplified with high efficiency in the multiplex (ftsZ = 94% and efficiency for 16S = 106%) as well as excellent linearity over the 7 orders of magnitude (r = 0.998). On testing AFB-positive bee, honey, and hive material, the 16S target typically amplified 3-5 cycles earlier (i.e. lower Cq's) than the ftsZ target as expected, due to the multiple copies of the 16S sequence per AFB bacterium compared to the single copy ftsZ gene.

The DNA extraction method from bees generated high purity DNA as shown by measurements on the microvolume spectrophotometer with OD260/280 measurements of between 1.8 and 1.9. Extracted DNA samples serially diluted in elution buffer showed the expected increase in Cq's with decreasing concentration, indicating no inhibitors were present in the samples following DNA extraction. DNA yields from swabs were too low to be measured on the spectrophotometer, however internal control Cq's for serial dilutions as for the bees, indicated the DNA was of suitable quality.

Upon extracting DNA from AFB positive material, it was serially diluted it in honeybee DNA and these dilutions used to compare the AFBduo assays with the conventional PCR tests described in the OIE protocols (Dobbelare, 1999; Govan, 1999). The AFBduo qPCR assay demonstrated two log greater sensitivity than the conventional PCR assays (data not shown).

In this study, we evaluated and compared the AFB levels among bees, the entire baseboard, and the entrance region of the baseboard. In the first cohort of

quarantine beehives from multiple regions in NZ (beehives from quarantine apiaries i.e. an apiary that had contained a hive exhibiting clinical signs), 45 hives were sampled up to 6 times during the 2018-2019 seasons. The samples taken were nurse bees, swabs from the entire baseboard and entrance swabs. Among these hives, 8 were found to have clinical signs of AFB ('roping' larval remains) during sampling while another 6 were presumed to have AFB due to their condition and the high prevalence in the apiary. Examples of the progression of AFB levels in three of the colonies is shown in Table 1, where the resulting Cq data has been converted to spores per 10 bee or swab sample processed.

In a second cohort of hives from one beekeeper, 23 hives were tested in a quarantine apiary using bee and hive entrance swab samples. Four of these hives showed clinical signs of AFB. Levels of AFB in nurse bee samples were compared to the levels indicated by the entrance swabs. Once again, most hives showed no or low levels of AFB, while those with clinical AFB demonstrated much higher spores levels of AFB in both the bee and entrance swab samples as shown in Table 2.

A third cohort of hives came from a beekeeper with a high incidence of AFB that was introduced through the purchase of contaminated equipment into his operation. Here we performed initial trials on pooling swab eluates into single extractions to reduce time and costs. Swab eluates were pooled into groups of eight and later twelve, the DNA extracted from the pooled sample and tested for the presence of AFB DNA. The remnant samples from pools that tested positive for both markers, were then tested individually as described. The results (Table 3) show that that even in the pools of twelve hive samples, single hives with higher levels of AFB could be clearly detected within a pool, prompting testing of those hives contributing to the positive pool to identify potentially clinically-affected hive(s).

Discussion

The AFBduo assay provided high confidence in the results due to the integrated internal control and detection of two markers for AFB. As expected, the 16S marker had approximately 10 to 20-fold higher analytical sensitivity due to the multi-copy nature of this marker. However the variability in Cq difference between the two markers among samples indicated the 16S copy number likely varied among AFB isolates and thus could not be relied on for quantification (Dahllof et al., 2000). This variability in copy number for diagnostic targets has been noted in other diagnostic qPCR assays for AFB (Rossi et al., 2018; Dainat et al., 2018). Therefore the use of the single copy ftsZ gene was used for quantification purposes using a standard curve prepared with a quantified synthetic template diluted in AFB-negative bee DNA.

Three sample types were compared in the first cohort – bees from the brood nest (nurse bees), swabs from the whole baseboard surface and swabs through the entrance of the hive only. The ability to sample the hive through the entrance removed the need to dismantle and disrupt the hive as with the first two sample types (bees and whole baseboard swabbing). The levels of both AFB and internal control DNA seen on the swabs taken from the whole baseboard or just the entrance area of the baseboard were similar (Table 4) possibly due to the maximum amount able to be collected on a given swab. This showed dismantling the hive to swab the whole baseboard was not required, in order to detect the higher levels of AFB in clinically-infected colonies. While the spore levels varied among clinical hives, they were still easily differentiated from the very low or undetectable spore levels in non-infected hives. While spore levels never increased during the monitoring time for many of the hives or became undetectable in some low spore cases, some of the

hives showed increasing levels at each testing point before clinical signs were observed in the respective hive and the hive destroyed (Table 1).

Spore levels were the highest in the bees from the brood nest area of the hive and while other studies have employed up to 100 bees per sample for culturing, the use of 10 bees permitted easier and more convenient handling for DNA extraction. Despite the lower spore amounts on swabs, the levels from the entrance swabs were still high enough to be differentiated from levels seen in hives not exhibiting any signs of AFB (Table 2). While most hives without clinical signs of AFB had low or undetectable levels of DNA detected, one hive (Hive 11) showed levels similar to two other hives with clinical AFB signs. Indeed, at the next inspection by the beekeeper (approximately two weeks after sampling), hive 11 showed clinical signs of AFB and was destroyed thus demonstrating the ability of qPCR to find infected hives that may have pre-clinical infections or have symptoms missed by visual inspection.

However these results also demonstrated the importance of knowing the history of the hives sampled. In two cases (hives 11 and 12 in Table 2), the bees showed high levels of AFB spores while the hiveware showed low or undetectable levels. Subsequent investigation showed that the hiveware was new (< 2 weeks old at time of sampling) for both these hives and that the colonies were newly-established from an unknowingly infected parent colony. Therefore, while the bees carried high levels of AFB there had not been enough time for spores to be deposited around the hive entrance surfaces. Further research will be required to assess this accumulation rate of spores on the hive.

Given the ability to detect clinically relevant levels of AFB through this simple hive entrance swabbing method, it is highly likely that the same DNA sample can be used

to test for other bee pathogens. *Nosema ceranae* has been detected through the use hive debris and qPCR (Copley *et al.*, 2012) and showed good correlation of levels between bees and debris. Since the development of this current work, a report has used hive debris collected over the period of a week to estimate loading of European foulbrood (a disease not present in New Zealand currently). As with the results described here, the bees offered a higher sensitivity of pathogen detection, yet the hive debris results still provided predictive information as compared to non-infected hives (Biová *et al.*, 2021). Another application could for biosecurity surveillance for unwanted hive pests such as tracheal mites (*Acarapis woodi*) or small hive beetle (*Aethina tumida*). The use of the sampling technique here combined with a qPCR assay (e.g. Li et al., 2018; Ward *et al.*, 2007) could provide rapid detection of any incursion to countries such as New Zealand where small hive beetle is not found, by providing faster sampling of hives than dismantling hives to extract hive debris for testing (Ward *et al.*, 2007). AFB has also been detected from winter debris using conventional PCR and thus quantification was not possible (Ryba et al., 2009). The quantification provides an important risk element of the hive - as to whether visual signs are likely present at the time of sampling or likely to demonstrate these clinical signs in the near future. In this work, levels of AFB spores of approximately 20,000 spores/swab or greater, were associated with either clinical or pre-clinical levels of the disease.

All the methods published to date have required either dismantling the hive to sample from the baseboard or repeated visits to the hive to insert and remove sampling sheets. In contrast, the rapid sampling method described here that we have named the Foster method is a fast, one-time sampling and quantification

method that provides clinically significant information as to the AFB status of the hive.

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Hive FL5

Date	14 Sept 2018	4 Dec 2018	16 Jan 2019	Clinical status
Bees (spores/10 bees)	-	24,448	779,266	Clinical signs seen 16 Jan after sampling and hive destroyed
Entrance (spores/swab)	ND	10,870	678,363	

Hive STF4

Date	22 Sept 2018	Clinical status
Bees (spores/10 bees)	2,591,937	Clinical signs seen early October 2018 and hive destroyed
Entrance (spores/swab)	4,281,312	

Hive RL02

Date	31 Oct 2018	27 Dec 2018	11 Mar 2019	23 Aug 2019	Clinical status
Bees (spores/10 bees)	ND	ND	ND	ND	No AFB seen
Entrance (spores/swab)	ND	ND	ND	ND	

ND Not Detected

\- Test not performed

Table 1: AFB spore levels seen in bee samples and entrance swabs from three quarantine hives examples followed over time. Total number of hives followed over time was thirty.

	Clinical signs of AFB?	Bees	Entrance Swab
		Spores/10 bees	Spores/swab
MLHive 1	**Yes**	**37,097,589**	**3,138,994**
MLHive 2	**Yes**	**514,061**	**24,008**
MLHive 3	No	ND	ND
MLHive 4	No	387	1,107
MLHive 5	No	539	377
MLHive 6	No	ND	ND
MLHive 7	No	80	ND
MLHive 8	No	210	ND
MLHive 9	No	205	ND
MLHive 10	**Yes**	**55,866,177**	**962,621**
MLHive 11	No**	9,518,751	ND*
MLHive 12	**Yes**	**12,561,091**	**890***
MLHive 13	No	<10	<10
MLHive 14	No	ND	<10
MLHive 15	No	2049	1,318
MLHive 16	No	ND	861
MLHive 17	No	ND	326
MLHive 18	No	ND	1,092
MLHive 19	No	97	207
MLHive 20	No	ND	11,235
MLHive 21	No	ND	326
MLHive 22	No	ND	201
MLHive 23	No	943,739	2,365

Table 2: Testing an apiary comparing bee and entrance swab samples. The spore levels marked with * indicate low level hive entrance spore on colonies recently placed into new hiveware (hives 11 and 12 were started from hive 10 splits). While at the time of sampling hive 11 showed no AFB symptoms (**), it developed clinical

symptoms 2 weeks later and was destroyed.

ND Not detected

	Pooled result (12) ftsZ spore levels	Individual Swab Result ftsZ spore levels (per swab)	Clinical Signs of AFB (following testing)
Hive SB216	**15,630**	Not detected	No
Hive SB217		Not detected	No
Hive SB218		Not detected	No
Hive SB219		**165,103**	**Yes**
Hive SB220		Not detected	No
Hive SB221		Not detected	No
Hive SB222		Not detected	No
Hive SB250		Not detected	No
Hive SB251		Not detected	No
Hive SB252		Not detected	No
Hive SB253		Not detected	No
Hive SB254		Not detected	No
Hive SB255	Not Detected	-	No
Hive SB256		-	No
Hive SB257		-	No
Hive SB258		-	No
Hive SB259		-	No
Hive SB260		-	No
Hive SB261		-	No
Hive SB262		-	No
Hive SB263		-	No
Hive SB264		-	No
Hive SB265		-	No

Table 3: composite pooling of swabs to rapidly find clinically relevant levels in hives. In this instance the first pool showed raised levels and the remnant swab eluates were tested individually, whereupon elevated levels were found in hive SB219. Subsequent hive inspections showed this hive to have clinical symptoms and it was destroyed.

– test not performed

Hive	Location	Estimated Spore level (ftsZ target)	Hive status
ML Shearer	Baseboard	52 million	Clinical signs of AFB
	Entrance	25 million	
ML Ellis	Baseboard	3.6 million	Clinical signs of AFB
	Entrance	3.4 million	
FL 10	Baseboard	61	No AFB seen
	Entrance	335	

Table 4: Comparison of baseboard and entrance swabs showing the differentiation of spore levels among two clinical hives and a hive without clinical symptoms.

Conflict of interest statement

JFM, REH and NTS are employees of dnature diagnostics & research Ltd., that developed the commercial AFBduo qPCR kit as well as the commercial bee DNA extraction kit method used in this study.

Acknowledgements and memoriam

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