Discovery of Lake Sinai Virus strains and *Lotmaria passim* trypanosome in New Zealand Bees

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Introduction

Discovery of pathogens such as viruses and bacteria has exploded with the advent of next generation sequencing. Due to the large bee losses reported in the USA and Europe over the past decade, increased attention and sequencing efforts have on honeybees (*Apis mellifera*) has uncovered new viruses and pathogens over the past few years.

Recently discovered viruses and pathogens include the re-classification of isolates of the trypanosome *Crithidia mellificae* to the newly named *Lotmaria passim* (Schwarz *et al.*, 2015) and the Lake Sinai Virus group (Runckel *et al.* , 2011). Since this first description of Lake Sinai Viruses 1 and 2, another 5 strains (LSV 3-7) have been described (Daughenbaugh *et al.*, 2015).

C. mellificae was described as a winter mortality agent in Belgium (Ravoet *et al.*, 2013) but these isolates are now classified as *L. passim*. It was also shown to cause increased mortality when in combination with the microsporodian pathogen *Nosema ceranae*. As we found both *Nosema ceranae* and *Nosema apis* to be present in extremely high levels in a recent bee mortality event (McFadden et al., 2015), we investigated whether *C. meliificae* or *L. passim* may also be involved.

Methods

As *C. mellificae* and *L. passim* had not been previously described in NZ, we developed novel qPCR assays targeting regions of the small subunit region (SSU) of *L. passim*, *C. mellificae* and *C. bombi* while avoiding similar regions for *N. ceranae* and *N. apis*. Primers and FAM-labelled hydrolysis probes were designed and synthesised (LGC Biosearch). In addition, primers flanking a region of variation between *C. mellificae* and *L. passim* were also designed for sequencing and high resolution melting, in order to be able to assign any positive samples (TrypanMelt-F and TrypanMelt-R) .

qPCR was performed in 10µl volumes on the Eco qPCR instrument (Illumina) and also the Mic qPCR instrument (BioMolecular Systems). Reactions used 300nM each primer and 150nM of probe and PerfeCTa qPCR ToughMix (Quantabio).

For the detection and resolution of Lake Sinai viruses, we used published primers that detected strains 1-4 (Ravoet *et al.*, 2015). We coupled these primers with high resolution melting to assess the strain diversity of any positive isolates. cDNA was synthesised from 5µl RNA (approximately 1µg RNA) using qScript cDNA supermix (Quantabio) following manufacturer's protocol. The resulting cDNA was diluted 1:5 with PCR-grade water and 2µl amplified on either the Eco or Mic instruments using 300nM primers LSV1-4-F-2157 and LSV1-4-R-2309 (Daughenbaugh *et al.*, 2015). Cycling conditions consisted of 95°C 2 minutes followed by 40 cycles of 95°C 5 seconds and 60°C 20 seconds, followed by melting from 65°C to 95°C and continuous fluorescent acquisition.

Results

High levels of target amplification were detected with the new trypanosome qPCR assays (Figure 1) with no cross-reactivity observed with *N. ceranae* or *N. apis*. In order to determine the target identity, ten positive samples were amplified with the primers TrypanMelt-F and R using AccuMelt (Quantabio) and high resolution melting as for the Lake Sinai Viruses described. All amplifications generated identical high resolution melting profiles (not shown) while sequencing of the amplicons indicated the region of sequence variation matched that of *L. passim* rather than *C. mellificae*.

The LSV primers generated positive amplification on cDNA samples and generated a number of melting profiles (Figure 2). Examples from each melting cluster were sequenced directly using the same LSV primers. Sequences obtained matched strains LSV1 (#HQ871931) and LSV3 (#JQ480620).

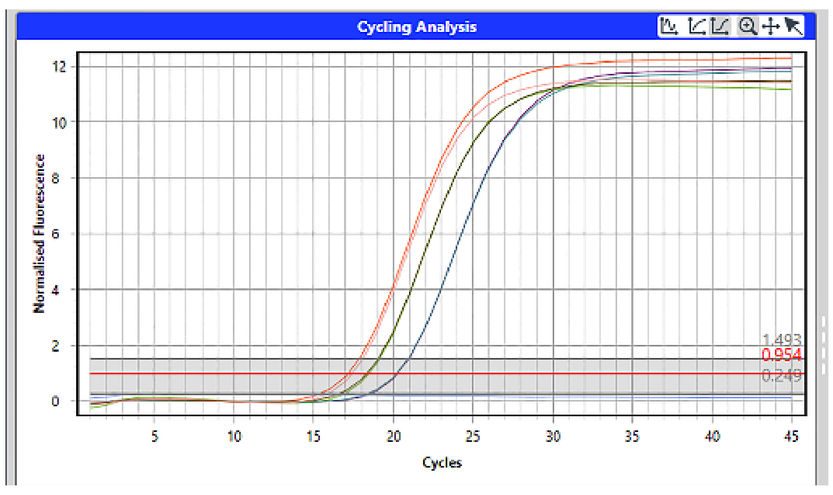


Fig 1: Amplification of Lotmaria passim
Amplification of 3 samples (in duplicate) with the novel trypanosome qPCR assay. High levels of *L. passim* were detected as evidenced by the Cq results of 10-20.

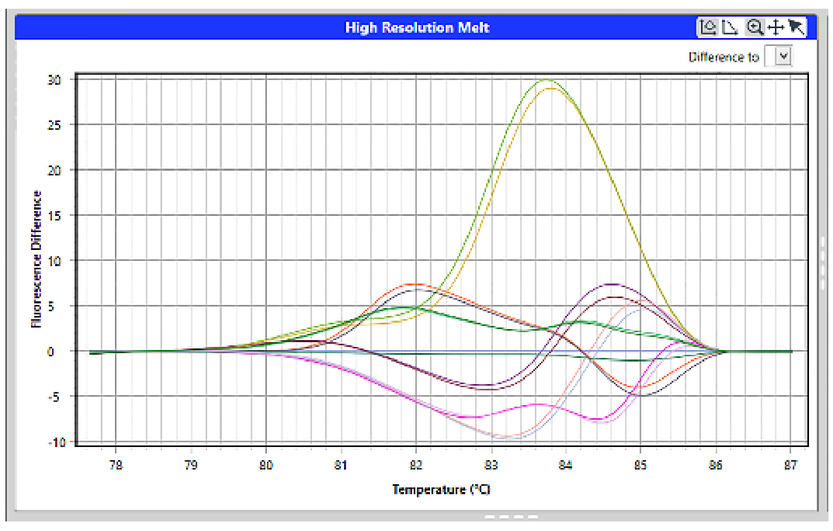


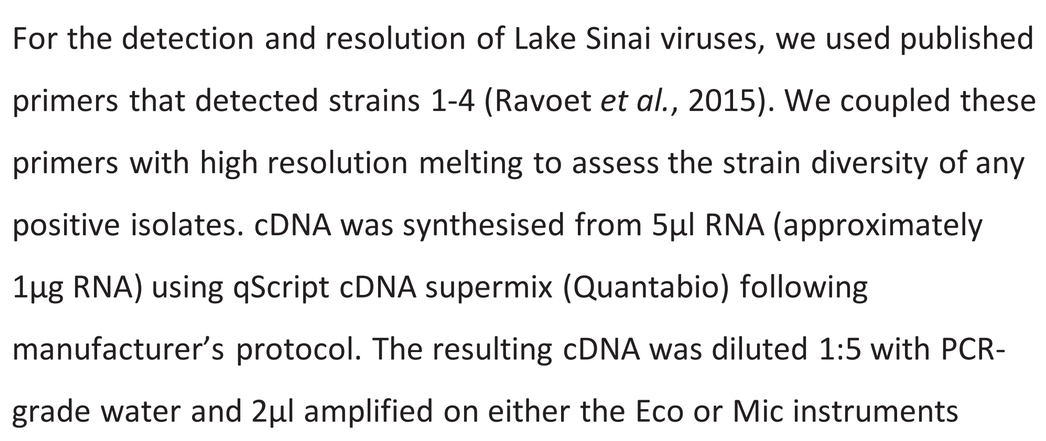
Fig 2: High Resolution Melting Analysis of Lake Sinai Virus strains
A number of cDNA samples were amplified in duplicate with primers detecting multiple LSV strains. High resolution melting plots show the genetic variation between samples

Discussion & Future Work

The design of new qPCR assays for trypanosomes and the use of existing primers together with our use of high resolution melting have confirmed the presence of both *Lotmaria passim* and several strains of the Lake Sinai Virus group in New Zealand.

Ongoing testing for *L. passim* has indicated that the trypanosome is widely-spread within New Zealand and has been here for at least 5 years (and likely much longer). While high levels of the trypanosome have been found in conjunction with both *Nosema ceranae* and *Nosema apis*, *Lotmarie passim* has also been seen without either nosema present. The effects of this agent alone require further work. It may be that the damage of the trypanosome to the gut of the bees allow faster access by subsequent pathogens, such as *Nosema ceranae*.

Lake Sinai Viruses have been shown to replicate in both bees and varroa mites. LSV2 levels have been associated with declining hives (Daughenbaugh et al., 2015) and also with nosema spore counts (Traynor et al., 2016). Current work is assessing the presence and levels of these specific strains in New Zealand.



Lotmaria passim trypanosome (R. Schwarz)

References

Daughenbaugh et al., *Viruses* 2015;7, 3285-3309;
McFadden A., Mackay J., Borowik O. & Goodwin M. 2016 Surveillance 43 (1)
Schwarz et al., *J Eukaryot Microbiol.* 2015;62(5):567-83
Ravoet et al., Plos One 2013;26;8(8):e72443
Runckel *et al.*, Plos One 2011;6(6):e20656
Traynor *et al.*, Apidologie 2016; 1-23

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